THE METABOLISM OF CARBOHYDRATE BY PLEOMORPHIC AFRICAN TRYPANOSOMES, AND THE MODE OF ACTION OF ARSENICAL DRUGS

by

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The pleomorphic strains have also been shown to differ from the monomorphic organisms in possessing ∝-keto-acid oxidase systems for the decarboxylation of pyruvate and ∝-oxoglutarate. These enzymes have been investigated and shown to resemble the oxidative decarboxylases of higher organisms. Despite the development of these enzyme complexes, the tricarboxylic acid cycle has been shown to have minimal activity in pleomorphic T. rhodesiense due to the limiting levels of citrate synthetase (E.C. 4.1.3.7.) and succinate dehydrogenase (E.C. 1.3.99.1.). However, carbon balance studies show that the induced formation of succinate in vitro probably occurs via the first steps of this pathway.

The utilisation of oxygen by the pleomorphic trypanosomes involves the L-∝-glycerophosphate oxidase system previously reported in the monomorphic organisms. No cytochrome pigments could be demonstrated in either form, although a study of the effects of respiratory inhibitors on the pleomorphic strain provides evidence for the existence of an alternative, though minor route for oxygen utilisation.

The rapidity of the trypanocidal effect of the arsenical drug melarsen oxide led to the hypothesis that the drug inhibits the supply of metabolic energy to the organisms. The metabolic studies presented indicate that glycolysis is the major pathway of energy production in the pleomorphic organisms, and the glycolytic enzyme pyruvate kinase ((E.C. 2.7.1.40.) (PK) has been found to be susceptible to inhibition by melarsen oxide both in vitro and in vivo. This enzyme is shown to be the major point of inhibition by this drug in vivo, and accordingly, trypanosome PK has been purified and its properties have been extensively investigated.

Trypanosome PK is a hydrophobic and exceptionally unstable enzyme which is allosterically activated by it's substrate phosphoenolpyruvate (PEP). The homotropic PEP binding interactions are abolished by the presence of the heterotropic activator fructose-1,6-diphosphate (FDP). It appears probable that trypanosomal glycolysis is maintained in an active state in vivo by the activation of PK by FDP. There is no control exerted on PK by other glycolytic intermediates or by ATP.

Kinetic investigation of the interaction of this enzyme with it's cosubstrates PEP and ADP, with FDP, and with melarsen oxide, has led to the proposal of a model to describe the molecular mode of action of drug. The inhibitory properties of the arsenicals appear to be integrally connected with the allosteric properties of the enzyme. The inability of melarsen oxide to inhibit the non-allosteric PKs of such host organs as heart, brain and skeletal muscle indicates that the possession by the parasites of this allosteric enzyme is instrumental in allowing the arsenicals to exert their trypanocidal effect. This hypothesis is borne out by the sensitivity of the allosteric PK of host liver to the arsenicals.

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Abbreviations.

The following abbreviations are used in the text.

PEP	phosphoenolpyruvate.
F6P	fructose-6-phosphate.
FDP	fructose-1,6-diphosphate.
G6P	glucose-6-phosphate.
L-α-GP	L-α-glycerol-3-phosphate. (see Hirschmann, 1960)
TEA	triethanolamine.
BSA	bovine serum albumin.
ICDH	isocitrate dehydrogenase.
MDH	malate dehydrogenase.
PK	Pyruvate kinase.
HK	hexokinase.
LDH	lactate dehydrogenase.
pCMB	p.chloromercuribenzoate.
BAL	British anti-lewisite = 2,3 dimercapto-1-propanol.
Lewisite	Dichloro (2-chlorovinyl) arsine.
RQ	Respiratory quotient.
TPP	thiamine pyrophosphate.
DTT	Dithiothreitol (Clelands Reagent).
CySH	Cysteine.
CyS.SCy	Cystine.
DHAP	Dihydroxyacetone phosphate.
FFA	Free fatty acids.
α-OG	α-oxoglutarate.
2-PGA	2-phosphoglycerate.
3-PGA	3-phosphoglycerate.
PCA	Perchloric acid.
EATRO	East African Trypanosomiasis Research Organisation.
TREU	Trypanosomiasis Research Edinburgh University.

CHAPTER I

GENERAL INTRODUCTION AND SURVEY OF THE LITERATURE - METABOLIC STUDIES.

1) THE LIFE CYCLE OF BRUCEI-GROUP TRYPANOSOMES.

The sub-genus Trypanozoon of the Salivarian trypanosomes (Hoare, 1964) includes T.brucei, the causative agent of animal trypanosomiasis in Africa, and T.rhodesiense and T.gambiense, the parasites producing human sleeping sickness. These organisms are characterised by the presence of multiple morphological forms in the bloodstream of the vertebrate host, and by the possession of a complex life-cycle involving further changes of form in the insect vector.

The bloodstream forms of these organisms show large variations in morphology, the relative numbers of the identifiable types being dependent upon the stage of the infection. When the parasitaemia is rising (relapse phase) the majority of the organisms are long (22-33 μ) and slender, possessing a free flagellum at the anterior end, whereas there is a high proportion of short (14-20 μ) "stumpy" forms when the parasitaemia is falling (remission phase), (Fantham & Thomson, 1911; Ashcroft, 1957). These "stumpy" forms appear to be produced from the slender organisms by the action of the host's immune response (Ashcroft, 1957; Wijers, 1960. See, however, Luckins, 1969), and are characterised by a greater cell diameter in the region of the nucleus, and by the absence of a free flagellum, although an undulating membrane is still present.

This multiplicity of form has led to the application of the term polymorphic to the infection (Hoare, 1956; Brenner, 1965); however, Oehler (1914) showed that as the variation in form persists in a cloned infection, the multiplicity can have no genetic basis, and hence the term pleomorphic is preferred in this work (Vickerman, 1965; Ormerod, 1967).

After ingestion of infected blood by the tsetse fly vector (Glossina spp.), further development of the trypanosomes occurs. It has been

demonstrated (Robertson, 1912; Reichenow, 1921; Wijers & Willett, 1960) that of the bloodstream forms, only the stumpy form develops in the insect mid-gut. In this partially anaerobic and cooler environment the stumpy forms multiply and elongate to give the epimastigote, or midgut form, which is similar to the bloodstream organisms in so far as the nucleus is still anterior to the kinetoplast. These flagellates migrate across the alimentary tract of the tsetse fly, and eventually attach to the walls of the salivary glands, where a transposition of nucleus and kinetoplast occurs to produce the crithidial form. Further multiplication and another reversal of the positions of nucleus and kinetoplast, gives rise to the metacyclic trypomastigotes. This stage of development is slightly smaller than, but morphologically similar to, the bloodstream stumpy form, and is the form which is infective to the mammalian host when injected with the fly's saliva.

Morphological changes on in vitro cultivation.

Flagellates of the Trypanozoon sub-genus are exceptionally difficult to establish in culture. Where a successful growth has been obtained (e.g. Ryley, 1962) it has been found that after inoculation of the cultures with the bloodstream trypomastigotes, the flagellates take on the morphological characteristics of the insect midgut form. This change has usually been paralleled by a loss of infectivity to both the tsetse fly and the mammalian host, but there are reports of infective organisms being obtained from cultures (Trager, 1959 (T.vivax); Weinman, 1957).

On the basis of the superficial resemblance between the culture forms and the tsetse fly midgut organisms, these cultured organisms have been used as the basis for biochemical investigations on that part of the life cycle occurring in the insect vector, to avoid the obvious difficulty in obtaining adequate amounts of experimental material directly from the

flies. However it is interesting to note that short term cultivation of the bloodstream forms has now been obtained, without the morphological switch to the vector epimastigote form occurring (Le Page, 1967), and this should allow a more detailed study of the factors influencing the morphological changes in these organisms.

The maintenance of strains in the laboratory

Trypanosomal infections may be transmitted in the laboratory either by cyclical passage through tsetse flies, or by direct syringe passage from host to host, and, due to the difficulties involved in maintenance of a tsetse colony the latter is the preferred method. However, after several passages, the pleomorphism of the strain gradually disappears, resulting in a monomorphic infection, morphologically identical to the slender form of the pleomorph (Fairbairn & Culwick, 1947; Ashcroft, 1960). These monomorphic strains no longer show the relapsing infections characteristic of the natural pleomorph, but produce a fulminating infection, killing the host in 3 - 4 days. At the same time the organisms can no longer be established in culture, and are not infective to the insect vector (Ashcroft, 1957), lending weight to the observation that it is the stumpy forms which complete the life cycle in the fly.

Thus the maintenance of pleomorphism is dependent upon the natural transmission via the tsetse fly, although the factors involved in this dependence are as yet unknown (Newton, 1968).

Mitochondrial changes associated with cyclical transmission.

Evaluation of the above stages in the life cycle of T.brucei, has been based on various characteristics of the individual organisms, including breadth, length, presence or absence of a free flagellum, and the relative positions of the kinetoplast and nucleus. In this study,

for example, a combination of the first three of these characteristics was used to determine the relative proportions of the different blood-stream forms in a sample of infected blood (see Chapter 3). However, much work has been done, mainly by Vickerman (1962a,b, 1963, 1965) on the ultrastructural changes of the mitochondrion associated with cyclical development, with a view to linking morphological to cytochemical changes in the organism. The monomorphic bloodstream form of T.brucei has a single mitochondrial tube, starting from the kinetoplast and running to the anterior end of the organism, with a shorter tube extending to the posterior. The epimastigote form has a much more highly developed system, with a complex network of mitochondrial tubes extending anterior to the kinetoplast, and a longer posterior chondriome. A further difference between these extremes, is the possession by the epimastigote of numerous well defined cristae in the intramitochondrial space, and the apparent lack of cristal development in the bloodstream monomorph.

Intermediate between these forms is the stumpy bloodstream form which has an expanded mitochondrial tubule, the transformation from slender to stumpy trypanosomes being also accompanied by the appearance of numerous cristae in the mitochondrial lumen, and occasionally by a division of the chondriome (Vickerman, 1965).

Intermediate bloodstream forms in pleomorphic T.rhodesiense.

Throughout this work, the assumption has been made that the blood-stream forms of this organism can be classified into discrete slender (LS) and stumpy (SS) groups. Many authors have included a third class, the intermediate or intermediate stumpy (ISS) with various definitions of the characteristics of this ISS form. Newton (1968) used length to differentiate the bloodstream forms, whereas Bishop (1967) defines the

ISS form as having a free flagellum of "intermediate length." Wijers, (1959) further divided the pleomorphic organisms into long and short intermediate forms because of differences between his data and the original drawings of Lady Bruce (Bruce, 1911).

The variations in the length and breadth of T.rhodesiense blood-stream forms, have in fact been shown to be continuous (Swellengrebel, 1911) and the concept of the existence of several discrete morphological forms is thus erroneous. However, from a cytological viewpoint, the ISS forms appear to be more closely related to the stumpy end of the morphological spectrum, than to the flagellate LS organisms (Vickerman, 1962a).

For the purposes of this investigation, a simple morphological division into slender and stumpy forms was used. Any organism lacking a free flagellum and conforming to a subjective estimation of increased nuclear width was classified as stumpy; all other organisms as slender. There appears to be a significant correlation between the increased width and the lack of the flagellum (see Figure 4). Also, this definition of the stumpy organism has a biochemical significance, (see Figure 6). Further definition of this classification will be found in Chapter 3.

2) CARBOHYDRATE METABOLISM OF BRUCEI-GROUP TRYPANOSOMES.

Studies on the metabolism of the bloodstream trypanosomes have been confined almost entirely to strains maintained for long periods in laboratory animals by syringe passage, and these monomorphic strains appear to differ biochemically as well as morphologically from the epimastigote form. As the intermediary metabolism of the epimastigotes (exemplified by the cultured organisms) has been fairly well documented, it seemed desirable to investigate the respiration of the stumpy form of the natural pleomorph, to determine the extent to which any changes have occurred prior to infection of the fly.

Metabolism of glucose by slender bloodstream forms

The motility of slender bloodstream forms of T.brucei and T.rhodesiense depends upon a continuous supply of exogenous carbohydrate (von Brand, 1933) which is metabolised at an exceptionally high rate. It has been calculated (Christophers & Fulton, 1938) that these organisms consume glucose equivalent to 50 - 100% of their own dry weight per hour, utilising O_2 in equimolar amounts, and producing very little CO_2 (Ryley, 1962). A radioactive carbon balance showed that glucose is metabolised by a classical glycolytic sequence without the intervention of the pentose phosphate shunt pathway (Grant & Fulton, 1957) and in the absence of a functional tricarboxylic acid cycle, (Marshall, 1948; Ryley, 1956) pyruvate is excreted, accounting for at least 83% of the glucose carbon utilised aerobically (Grant & Fulton, 1957). Earlier measurements of the end products of glucose metabolsim by monomorphic trypanosomes, led to some confusion on this point. Grant and Fulton found 10% glycerol and a trace of succinate along with the large pyruvate yield, and Ryley(1956) again found pyruvate to be the major end-product (78%) with 19% glycerol, 2% CO_2 and a trace of succinate. Shaw et al., (1964) found 60% alanine and 40% lactate as the products of glucose metabolism, but these data are based solely on recovered radio-activity. No pyruvate was estimated and their organisms were contaminated with WBCs, making these figures totally unreliable. Fulton and Stevens (1945) found, in addition to these products, acetate, formate and ethanol in a qualitative experiment, and Glowazky (1937) found oxalate to be produced. However, with the demonstration by Dixon (1966) that platelet contamination of trypanosome preparations is entirely responsible for the apparent LDH activity of these cells, previous work showing or implying lactate as an end-product appears to be in some doubt (Shaw et al., 1963; Fulton & Stevens, 1945; Ryley, 1962). Present opinion seems to be that the only significant products of aerobic metabolism of glucose are pyruvate and glycerol with

a trace of succinate, although von Brand (1952), differentiating between T.brucei and T.rhodesiense, states that glycerol is only produced anaerobically in T.brucei.

The mechanism of O_2 utilisation by slender bloodstream forms.

No detectable cytochromes have ever been shown to be present in these organisms (Grant et al., 1961; Ryley, 1956,1962; Fulton & Spooner, 1959) and their glucose metabolism is insensitive tc cyanide and azide (Ryley, 1956) and carbon monoxide (Fulton and Spooner, 1959). Despite the apparent lack of these pigments, T.brucei metabolising glucose supports a very high rate of oxygen uptake, and some other system must be involved in reoxidation of glycolytic NADH, in the absence of LDH.

Molecular oxygen is used as a terminal oxidant without the intervention of a conventional electron transport chain, by an L.∝. glycerophosphate (L.∝.GP) oxidase system (Grant & Sargent, 1960) containing iron, thiol groups and possibly FAD (Bide and Grant, 1964; Grant and Sargent, 1961). The complex therefore differs from the oxidases present in brain mitochondria (Ringler and Singer, 1959) and in insect muscle sarcosomes (Estabrook and Sacktor, 1958a,b) which use the cytochromes to mediate electron transfer from L-∝-GP to oxygen. The trypanosome system is extra-mitochondrial but particulate (Bide, 1963), and consists of a coupled aerobic dehydrogenase and peroxidase (Bide 1963). This system, in the presence of a soluble NAD-linked ∝-GP dehydrogenase allows reoxidation of glycolytic NADH according to the following scheme:

(Grant & Sargent, 1960)

$$H^+ + DHAP + NADH \longrightarrow L\text{-}\alpha\text{-}GP + NAD^+$$

$$L\text{-}\alpha\text{-}GP + \tfrac{1}{2}O_2 \longrightarrow DHAP + H_2O$$

$$\text{Sum: } H^+ + NADH + \tfrac{1}{2}O_2 \longrightarrow NAD^+ + H_2O$$

The particulate oxidase is insensitive to inhibitors of electron transport in higher organisms, such as cyanide, azide, amytal, and antimycin A (Grant & Sargent, 1960) and the data of these authors show that this system is sufficiently active *in vitro* to account for at least 85% of the O_2 consumption *in vivo*. However in their discussion of this enzyme complex, they do assume that this is the only hydrogen transport system present in the organism.

Metabolism of glucose by culture forms.

Whereas the slender bloodstream forms of *T.brucei* are characterised by an incomplete oxidation of glucose and the concomitant accumulation of glycerol and pyruvate, the cultured epimastigote forms carry the oxidation to completion, producing CO_2 and little organic acid (Ryley, 1962). Spectroscopic examination of the culture forms by the same author showed the presence of absorption bands characteristic of cytochromes a and b, but no evidence was found for the presence of cytochrome c. Aerobic glucose oxidation by whole cells, was markedly sensitive to cyanide, indicating the presence *in vivo* of a functional electron transport chain.

The oxidation of L.∝GP is much slower in homogenates of the epimastigote than in lysates of slender bloodstream forms (Grant *et al.*, 1961) although no evidence was presented as to whether this substrate was utilised by the cytoplasmic oxidase or by a mitochondrial system linked to the cytochrome chain. However, the cyanide *insensitive* oxidation of NADH by frozen and thawed cells (Grant *et al.*, 1961) indicates that the cytoplasmic oxidase may still be operating, although the oxidation of L∝GP in the same system *was* cyanide sensitive. This anomaly cannot be explained on the data presented by these authors.

Enzymes of the tricarboxylic acid cycle in bloodstream and culture forms.

Although the overall metabolism of glucose by these two forms differs markedly, and the slender bloodstream forms appear to be incapable of utilising the tricarboxylic acid cycle, several of the enzymes involved in this cycle are common to both stages of the life cycle (Ryley 1962). Malic dehydrogenase (E.C. 1.1.1.37), aconitase (E.C. 4.2.1.3.), fumarase (E.C. 4.2.1.2.) and isocitric dehydrogenase (E.C. 1.1.1.42), are all found in the slender organisms, but in every case at a much lower activity than in the epimastigote (Ryley, 1962) although these results may be due to the assay temperature being between the natural environment temperatures of these organisms (37°C and 25°C respectively). No evidence has been presented for the presence of oxidative decarboxylation systems in the slender forms (Ryley, 1962), nor has succinic dehydrogenase activity been detected.

This same author showed by the spectrophotometric method of Korkes (1962), the presence of an active pyruvate oxidase in the culture organisms, and demonstrated that whole cells of this form can metabolise succinate, this activity being more marked at slightly acid pH. Unfortunately, although this work gives an excellent outline of the enzymic competence of the bloodstream and culture organisms the quantitative aspects are in some doubt since LDH was shown to be present in the enzyme preparations. However, the apparently complete oxidation of glucose to CO_2 with an R.Q. approaching 1, indicates that in parallel with T.gambiense culture forms, a fully functional tricarboxylic acid cycle is operative at this stage of the life cycle.

Metabolism of glucose by stumpy bloodstream forms.

Despite the relatively large amount of data available on the glucose metabolism of the artificially induced monomorphic trypanosomes, the amount of work done on a true pleomorphic strain has been minimal.

At some point in the natural development of the trypanosome from slender bloodstream to mid-gut epimastigote form, the metabolic complexity must change from the simple glycolytic system to the complete oxidative capacity of the tsetse mid-gut form. To what extent these changes occur prior to infection of the insect vector was practically uninvestigated.

Grant and Fulton (1957), investigating the metabolism of a newly isolated pleomorphic strain, found that pyruvate production and labelling from 1-^{14}C glucose was identical with that of an old monomorphic strain. However, whereas none of the tricarboxylic acid cycle intermediates sustained respiration of the monomorph (Ryley, 1962), Balis (1964) showed that motility of the culture forms was retained with α-OG as an energy source. This observation became more significant when Vickerman (1965) showed that in a pleomorphic strain, the motility of the stumpy forms was preferentially supported by this substrate. This was the first evidence contrary to the suggestion of Grant et al., (1961), that the respiratory switch from aerobic glycolysis to classical aerobic respiration mediated by cytochromes and a tricarboxylic acid cycle, occurred only on transfer to the lowered oxygen tension available in the insect mid-gut or in culture. This induction of cytochrome synthesis and oxidative enzymes has been shown to occur with a decrease in available oxygen in Pseudomonas sp.(Lenhoff et al., 1956). Further evidence that the switch may be partially effected on transformation to the stumpy bloodstream forms came from cytochemical studies (Vickerman, 1965) involving the deposition of formazan when fixed trypanosomal smears were incubated with a tetrazolium salt and NADH, as a cytochemical indicator of the presence of "diaphorase" in the organism. In T.vivax and

T.congolense which are thought to have a complete oxidative metabolism in their bloodstream forms (Ryley, 1956), deposition of the formazan was dense and was located in the mitochondria. When a pleomorphic strain of T.brucei was tested, the formazan deposits were confined to the intermediate and stumpy forms, the slender trypanosomes showing no mitochondrial formazan deposits. As further evidence that these slender forms are analogous to the monomorphic syringe-passaged strains, tests on the latter also gave no mitochondrial formazan deposition.

Newton (1968) in reviewing this work, replaced Vickermans non-specific term "diaphorase" with a specific enzyme title, "NADH - lipoamide oxido-reductase," and extrapolation not justifiable from the original results. This enzyme, catalysing the transfer of electrons from the reduced coenzyme to a tetrazolium salt is probably best described as a "tetrazolium reductase," and one of these has been shown to be a flavoprotein which is probably autoxidisable (Nachlas et al., 1958). In view of the number of flavoproteins that catalyse the reduction of intermediary metabolites by NADH, and the ability of reduced flavoproteins to reduce tetrazolium salts, it is obvious that to ascribe the results of Vickerman to a particular enzyme is illogical. This is borne out by the fact that in the same report (Vickerman, 1965) the author found extra-mitochondrial formazan deposits in the slender bloodstream form, which he attributed to the action of the L.α.GP oxidase system, shown to be a cytoplasmic, particulate flavoprotein (Bide, 1963).

Conclusion.

It appeared that the metabolic switch must therefore be at least partly stimulated by some event occurring in the bloodstream of the mammalian host. As suggested by Newton (1968), the assumption of an

increased oxidative capacity by the stumpy form may explain the preferential survival of these organisms in the tsetse mid-gut, and the apparent inability of the slender form to infect the insect vector. As the only evidence for this diversity of metabolism in the blood-stream forms was on the basis of a motility test and the qualitative, non-specific tetrazolium reductase analyses, it seemed desirable to investigate further the biochemical changes which have occurred before infection of the tsetse fly, despite the negative results of Grant and Fulton (1957). Previous reports on the aerobic metabolism of monomorphic T.brucei have suffered from a degree of inconsistency, probably due to differences in preparative methods. Whereas removal of erythrocytes and leuocytes from infected blood is relatively easy, successful removal of platelets does not seem to have been achieved in many cases. The method described by Grant and Fulton (1957) for prep. of monomorphic trypanosomes, in my hands yielded preparations with a high platelet content, as measured by LDH activity. Similarly the repeated centrifugation technique reported by Ryley (1956,1962), whilst completely eliminating RBC's and WBC's, did not yield trypanosomes free from LDH contamination. Other studies based on the comparison of infected and uninfected blood (e.g. von Brand et al., 1950) are suspect, due to the altered blood element picture obtained in infected animals, which show a marked polythrombocytaemia. As a result, the basic metabolic studies carried out on the pleomorphic strains in this work, were repeated both on an established monomorph, and on a pleomorphic strain in which monomorphism was freshly induced by syringe passage. The isolation method described in Chapter III section 1 gave trypanosome preparations completely free from blood elements.

3) THE ACTION OF ARSENICALS ON METABOLISM.

Compounds of arsenic have been used for hundreds of years, for both medicinal and homicidal purposes, and as a result much work has been done with a view to elucidating the mechanism of action of these compounds. Early work was concentrated on the effects of inorganic arsenite and arsenate, the interconversion of these forms, and their relative toxicities. It was not until 1905, more than 60 years after the discovery of the first organic arsenical, that Thomas and Breinl realised the value of sodium arsanilate in murine trypanosomiasis, and at the same time Koch showed this compound to be trypanocidal in human sleeping sickness. These observations were contrary to the findings of Ehrlich, who had previously shown that sodium arsanilate was inactive *in vitro* against trypanosomes, but they led to the introduction of many organic arsenicals as trypanocides, and also as effective agents against amoebiasis and syphilis.

By this time, the mid-nineteenth century concept that arsenicals were "protoplasmic" poisons with a non-specific coagulating action, had been firmly replaced by the idea of their interfering with cellular metabolism, and Ehrlich (1909) developed this concept further. He demonstrated that the arsenicals do not act by release of inorganic arsenite, and that the pentavalent arsenicals are probably reduced *in vivo* prior to their activity against the cell, but the major importance of this work lay in his formulation of the concept of specific arseno-receptors. These specific receptor groups within the cell were postulated to be thiol groups (Voegtlin *et al.*, 1923), and Barber (1929) showed that condensation can occur between phenylarsenoxides and organic thiols such as thiolacetic acid and thioglycollic acid.

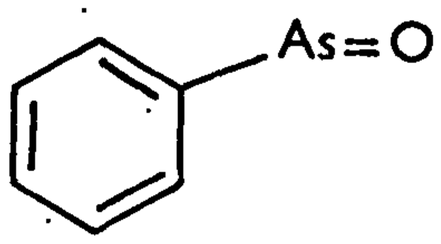
From this demonstration, it was to be expected that in the presence of a large excess of exogenous thiol, the toxicity of the arsenicals would be much reduced. This was experimentally proven by Eagle (1939) who showed that the anti-spirochaetal activity of trivalent arsenicals is abolished by free thiol compounds such as cysteine, but not by methionine. Unfortunately, although these data from protection experiments were held for many years as circumstantial evidence for the involvement of thiol groups in arsenical action, they are in fact completely worthless in this respect. Such data only indicate the relative binding affinities of the cellular receptor whatever its nature, and the exogenous thiol for the arsenical.

However, much work has accumulated since the postulate of Voegtlin et al., (1923), to point to free thiol groups as the arseno-receptors of Ehrlich. Krebs (1933a,b), by demonstrating the accumulation of keto-acids such as pyruvate in kidney cells treated with arsenite, gave the first indication of the primary metabolic target. It became evident that the oxidation of keto-acids is the key reaction in arsenite inhibition, and intensive study of the organic trivalent arsenicals by Peters (Peters et al., 1945; Peters, 1955) and others showed that pyruvate oxidase is specifically and markedly inhibited by these compounds, presumably due to the involvement of lipoic acid and dihydrolipoyl dehydrogenase in the reaction mechanism. Many other enzyme systems have, however, been shown to be affected by the arsenicals, which are now used as common reagents for inhibition of thiol dependent reactions.

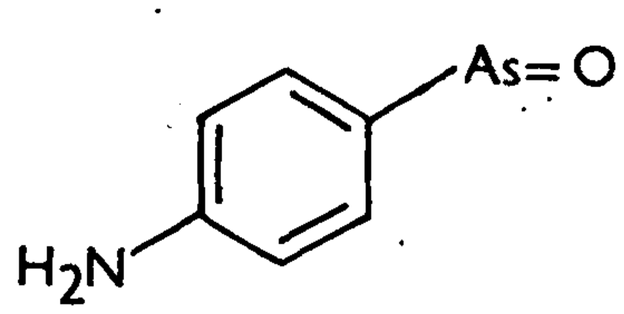
Despite the early introduction of the organic arsenicals, there is relatively little information on their metabolic effects compared to those of inorganic arsenite, although the inhibitions by these types of compounds often differ markedly. Although arsenite is trypanocidal,

Fig . I.

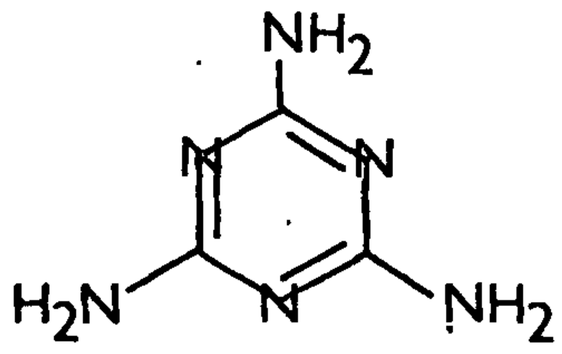
Melarsen oxide and derivatives.



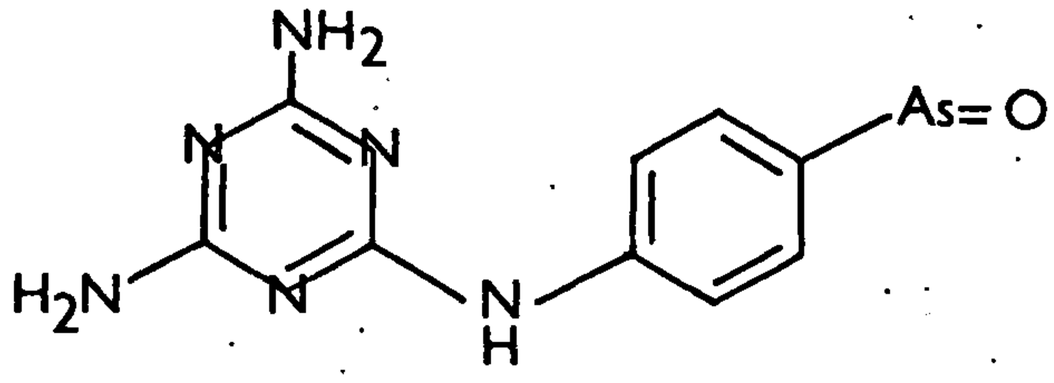
phenylarsenoxide.



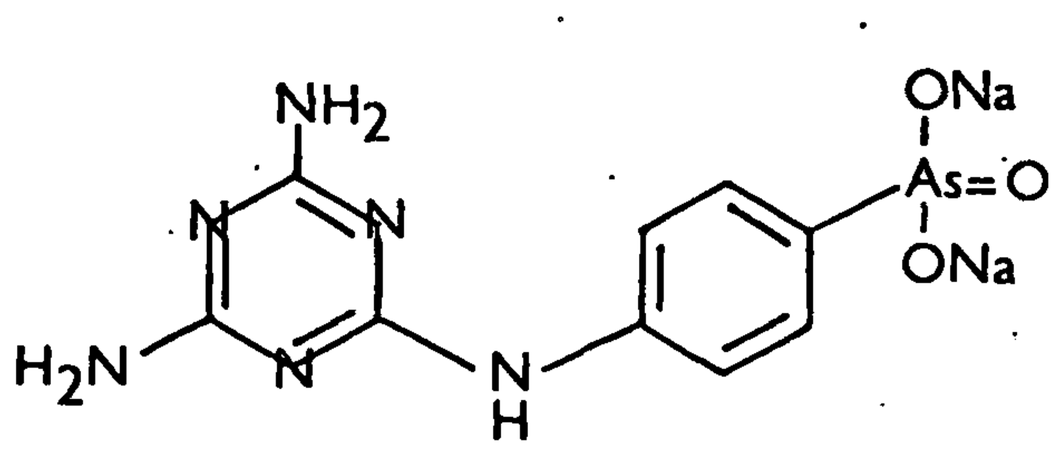
p aminophenylarsenoxide.



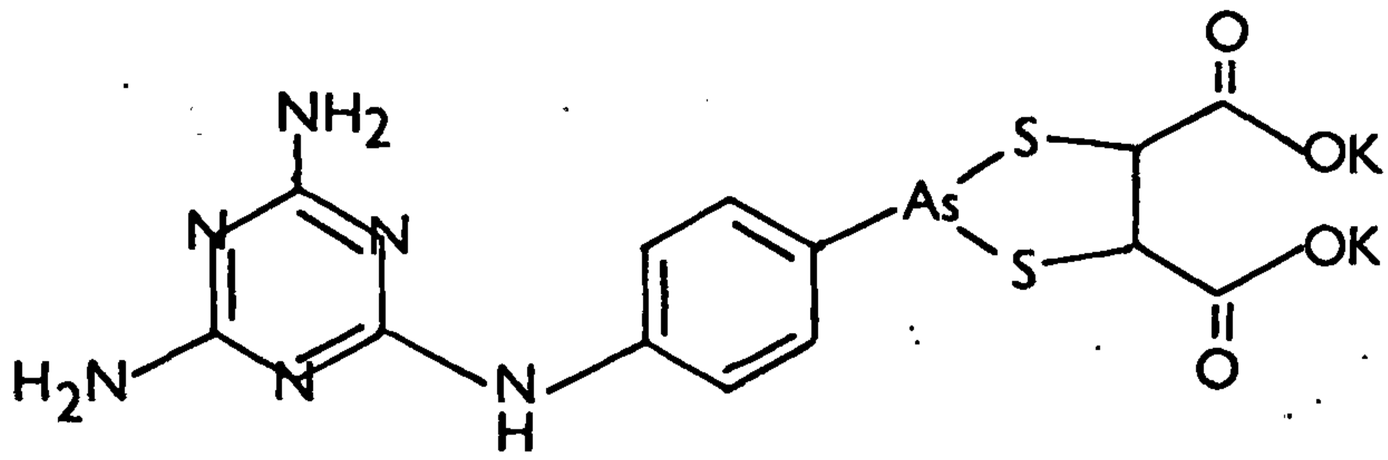
Melamine.



Melarsen oxide.



Sodium Melarsen (Mel 5).



Melarsonyl Potassium (Mel W).

its toxicity precludes its medical use. The relevant parts of this work will therefore be devoted in the main to a consideration of the aromatic arsenicals, especially as, to quote Webb (1966) "... there is a paucity of good delineation of the overall effects of the (organic) arsenicals on the metabolism of any organism or tissue, and essentially no studies bearing on the specificity of action in living tissue have been made."

Nomenclature.

The terminology used in this work will be that of Doak and Freedman (1960), although alternative names are available for most of the compounds involved. The arsenicals used are shown in Fig.1; for convenience the -As=O form is used in the text, but this is not intended to indicate the nature of these substances in solution, (see Webb, 1966).

Redox state of arsenicals in vivo

In 1947, Crawford stated that the supposed activation by reduction of pentavalent to trivalent arsenicals, was based entirely on indirect evidence, with no demonstration having been given of this occurring in the tissues. As a result he determined the pattern of excretion products, when the two forms of the phenyl arsenical were injected into rabbits (Crawford & Levy, 1947). Surprisingly he found that the expected reduction did not occur to any marked extent. Whereas over 90% of the pentavalent form was recovered *per se*, the major excretion product of the trivalent form was the oxidised compound. Frost (1967) cites numerous examples of this same phenomenon although his dogmatic statement that "Nature apparently may not produce trivalent organic arsenicals" is probably inaccurate.

Furthermore, the much lower toxicity of the pentavalent compounds indicates that reduction is at best a very much slower reaction than was thought, if it happens at all. The toxic side reactions of the trivalent forms, such as liver dysfunction and dermatitis, are not produced to the expected extent even in cases of chronic administration of the pentavalent compounds. Instead the pentavalent arsenicals often present a totally different clinical picture, causing optic nerve atrophy and resultant blindness.

The lack of knowledge of the selective permeability of cells to the arsenicals in vivo and the systems involved in the redox changes of the compounds, leaves some doubt as to the exact state of the arsenicals in the body. The selective action of the pentavalent compounds on the visual system may be due to differential permeability of the tissue or to a specific action of the drug itself. However, the vast majority of enzymes which have been investigated show no affinity for the pentavalent compounds. As will be shown in this work, there is a definite preference for the trivalent form as an inhibitor of PK which has been pinpointed as a major focal point in the trypanocidal activity of the arsenicals.

Development of melaminyl arsenicals

In the development of arsenical drugs, problems have been encountered with the onset of resistance to the drugs, and with permeability barriers. Aliphatic phenylarsenoxides and their reduced counterparts such as tryparsamide and orsanine have been found to be ineffective against T.rhodesiense although useful against the related T.gambiense. The latter species has been shown to develop resistance to tryparsamide very effectively, and thus some other derivatives were needed to replace this drug. Friedheim (1940) introduced a new class of trypanocide in

which a 2,4,6 - triamino-S-triazinyl (melaminyl) residue was attached in the para position in phenyl arsonate. This compound, known as melarsen was found to be very effective in the treatment of tryparsamide resistant strains (Williamson & Lourie, 1948), to be active as a trypanocide against T.rhodesiense, and to be useful in the treatment of advanced cases of the disease, where central nervous system involvement requires a drug capable of crossing the "blood-brain barrier."

The trivalent equivalent of melarsen, melarsen oxide, was found to be too toxic for direct use, and a derivative was prepared following the introduction of BAL (Peters et al., 1946) in detoxifying oxophenarsine (Peters & Stocken, 1947; Friedheim & Vogel, 1947). This combines the trivalent As in a stable 5-membered ring form with the two thiol groups of BAL, producing the cyclic thiol derivative, Mel B. This and melarsen are the only drugs effective against the late stages of T.rhodesiense infection (Hutchinson & Watson, 1965). The preparation of a water-soluble analogue of Mel B, Mel W, in which the As is coupled to dithiosuccinate proved disappointing against this trypanosome (Hutchinson & Watson, 1965), and apart from easier administration, this drug has no apparent advantages over Mel B or melarsen.

It is generally accepted that these disulphide derivatives are hydrolysed to the arsenoxide form prior to exerting their therapeutic effect, and the parent arsenoxide melarsen oxide has therefore been used in much of this present work.

Mechanism of trypanocidal action.

As previously mentioned the primary sites of organic arsenical drug action are often considered to be the ∝-keto-acid oxidases, but there is little convincing evidence to support this hypothesis. Of 109

references quoted by Webb (1966) on the inhibition of respiration and build up of keto-acids in the presence of arsenicals, 5 are studies of aromatic arsenicals and the rest are of lewisite and arsenite. Throughout his monumental work on the arsenicals as a class, Webb is continually and quite correctly lamenting the sparsity of data on the more complex aromatic arsenicals, compared to the volume of work on inorganic arsenite.

This is particularly relevant to the problem of trypanocidal activity where the monomorphic bloodstream forms have no active α-keto acid oxidase systems, and yet are quickly and strongly immobilised by the arsenicals. In these organisms, then, some other focal point must be the site of action of these drugs, and yet there has been little reliable work carried out to determine the nature of this receptor site. Much energy has been expended (Eagle, 1945; Eagle & Doak, 1951; Eagle et al., 1942, 1943, 1944a,b) on evaluating the effects of substitution on phenylarsenoxide toxicity and trypanocidal activity, and on the estimation of lethal and toxic doses of the drugs (Yorke et al., 1931; Fulton & Yorke, 1943; Kerr et al., 1963) but the active receptor site remains very much a mystery. Chen (1948) and Marshall (1948) produced some evidence that in T.equiperdum and T.evansi respectively, hexokinase is the most sensitive site in vivo. However, Cantrell (1951, 1953) came to the conclusion that this was not the case, although he used a comparison between infected /mapharsan treated and infected/untreated blood, with obvious difficulties of interpretation. Thirty minutes after treatment of one of a pair of rats with mapharsen, glucose utilisation and pyruvate production by the defibrinated parasitised blood samples were measured. Glucose utilisation rates were found to be identical in the treated and untreated samples, although pyruvate production was lower in the drug-treated blood, implying a site of action between glucose-6-phosphate and pyruvate.

Thus, from known actions of the arsenicals and the lack of pyruvate oxidase in bloodstream monomorphic T.brucei and T.rhodesiense, it is unexpected that arsenicals inhibit their respiration at all, and the mechanism of this inhibition was very obscure. It was therefore decided to investigate the mode of action of melaminyl arsenical drugs in parallel with an examination of the metabolic properties of pleomorphic T.rhodesiense.

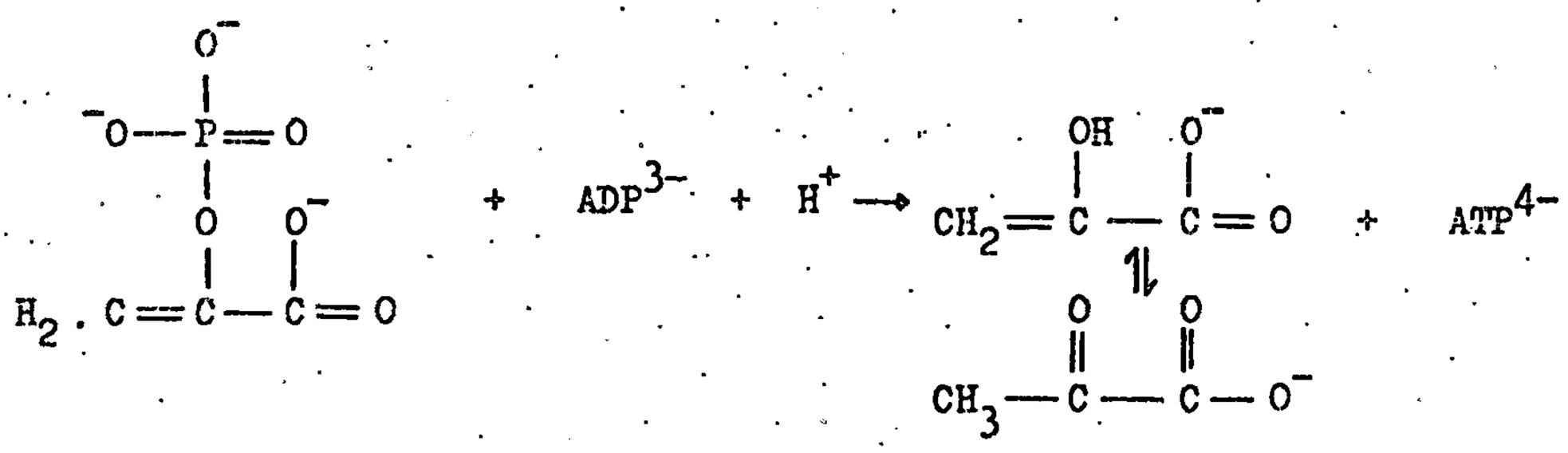
CHAPTER 2

GENERAL INTRODUCTION AND SURVEY OF THE LITERATURE - PYRUVATE KINASE.

1) GENERAL PROPERTIES

Chapter 4 of this work outlines the evidence leading to the conclusion that, *in vivo*, one of the primary arsenical-sensitive sites in *T.rhodesiense* is pyruvate kinase. Some sensitivity of hexokinase was observed, and a third site of action of these drugs is known, viz. glycerokinase (E.C. 2.7.1.30.) (Fairlamb, unpublished communication). The common denominator in the catabolism of glycerol and glucose by these organisms is thus, with respect to arsenical action, pyruvate kinase. The introduction to the enzymological part of this thesis will therefore deal with the properties of the mammalian PK's, to enable a comparison between host and parasite enzymes to be made from a chemotherapeutic and a kinetic viewpoint.

PK catalyses the transfer of a phosphoryl group from PEP to ADP, the enol form of the pyruvate product tautomerising spontaneously to the more stable keto form on release of the constraint imposed by the phosphoryl group on PEP.



PK has now been identified with the rabbit muscle enzyme which catalyses a CO_2-dependent phosphorylation of fluoride by ATP (Tietz and Ochoa, 1962) and with the enzyme from the same source which phosphorylates hydroxylamine (Kupiecki and Coon, 1959). The physiological significance of these reactions is still doubtful.

The existence of a new glycolytic intermediate between 3.PGA and pyruvate was demonstrated by Lohmann and Meyerhof (1934) who simultaneously purified PEP and elucidated its properties and molecular structure.

The role of the adenine nucleotides in the enzyme reaction was, however, unclear at this stage. Lohmann and Meyerhof were of the opinion that the reaction was one of dephosphorylation, with the coenzyme nucleotide acting as a catalyst for inorganic phosphate formation. Subsequently, AMP was postulated as a phosphoryl acceptor in the PK reaction (Needham and van Heyningen, 1935) but the presence of adenylate kinase in the enzyme preparations complicated the conclusions. Later work by Boyer et al., (1942, 1943) confirmed the suggestion of Meyerhof and Junowicz-Kocholaty (1942) that the nucleoside diphosphate was the physiological phosphate acceptor in the formation of pyruvate from PEP.

Cofactor requirements.

PK has in common with other kinases, a requirement for a divalent cation for activity, first demonstrated by Lohmann and Meyerhof (1934). Melchior, (1965) showed that $MgADP^-$ is the specific coenzyme required by PK, free ADP^{3-} not acting as a phosphoryl acceptor in the reaction. This requirement for a divalent metal ion may be met by various ions other than Mg^{2+}. Solvonuk and Collier (1955) showed that Mn^{2+} and Co^{2+} ions are utilisable, whilst Zn^{2+} and Sr^{2+} are ineffective in the red cell system.

Whereas a requirement for Mg^{2+} ions or a similar divalent cation is a general property of kinase enzymes, PK also shows a strict requirement for a monovalent cation. Boyer et al., (1942, 1943) demonstrated enzymic dependence on K^+ ions, (the first known role of K^+ ions as an enzymic cofactor) and Rb^+ or NH_4^+ ions have since been shown to replace this cation effectively (Kachmar & Boyer, 1953). Kinetic data (Kachmar

& Boyer, 1953), immunoelectrophoretic data (Sorger et al., 1965), NMR studies (Mildvan & Cohn, 1964) and UV difference spectra of tryptophan perturbation in the enzyme (Kayne & Suelter, 1965) have all been interpreted to mean that monovalent cations affect the conformation of the protein, and hence modify the binding of substrates. One report, however, claims that the enzyme from E.coli is unaffected by K^+ ions (Maeba & Sanwal, 1968a). The last steps in the purification procedure employed by these authors are ammonium sulphate extractions, and no indication is given that the NH_4^+ ions were removed prior to assay of K^+ activation. It is therefore possible that a sufficient concentration of NH_4^+ ions was added to the assay system to saturate the enzyme, prior to addition of excess K^+ ions.

Purification and physical properties.

PK is now accepted as being an essential component of glycolysis, and as such will occur in all tissues and organisms capable of utilising this metabolic pathway. The enzyme has been identified in most animal phyla, from protozoa to mammalia (see Boyer, 1962) as well as in many plant tissues (Miller and Evans, 1957) moulds, (Damodaran et al., 1955) and yeasts (Washio & Mano, 1960).

The first purification data, from rat muscle by Negelein (quoted in Bücher & Pfleiderer, 1962) were lost at the end of World War II, and the first published purification is that of Kubowitz & Ott from human muscle (1944). Crystallisation of the enzyme from various muscle sources has now been effected, and the yeast PK has been obtained in a high degree of purity (Haeckel et al., 1968; Hunsley & Suelter, 1969; Washio & Mano, 1960). Erythrocyte PK has been obtained in a highly purified state (Ibsen et al., 1968), and one hepatic isoenzyme (L_1) has been crystallised (Tanaka et al., 1967a).

The amino acid composition of PK from Saccharomyces sp. has been reported (Hunsley & Suelter, 1969; Bischofberger et al., 1970), and the mol. wt. of this enzyme was estimated at 185,000 - 200,000 (Bischofberger et al., 1970) and 138,000 - 166,000 (Hunsley & Suelter, 1969). Kuczenski and Suelter (1970) reported the molecular weight as 162,000 - 168,000 and further showed it to be a tetramer of four sub-units of approx. equal molecular weight of 42,000 - 45,000. This last figure corresponds to Büchers estimate of the molecular weight of Negeleins rat muscle enzyme of 166,000 (Bücher and Pfleiderer, 1962) obtained by a light scattering technique. The enzyme from rabbit muscle has been reported as being of molecular weight 237,000 (Warner, 1958), dissociating into 2 sub-units in 6M-urea (Morawiecki, 1960); each sub-unit consists of 2 polypeptide chains of 57,000 (Steinmetz and Deal, 1966).

Thus both yeast and muscle PK have been shown to consist of four chains. The leucocyte and erythrocyte enzymes, under the ultra-centrifugal conditions of Koler et al., (1964), have a molecular weight in the region of 150,000, and these enzymes also contain more than one sub-unit. PK possesses only two PEP binding sites (Reynard et al., 1961) and two Mn^{2+} binding sites (Mildvan and Cohn, 1965) per native muscle enzyme molecule of weight 237,000. This and other evidence has led to the now accepted model of PK being a tetramer containing two protomers or catalytic particles, each protomer being capable of subdivision into two non-identical polypeptide chains.

This concept of non-identical sub-units is consistent with the possibility of a regulatory function being ascribed to liver PK, in that the enzyme may consist of catalytic and regulatory sub-units as does aspartate transcarbamylase (Gehrhart & Schachmann, 1965). ATP has been reported as inhibiting PK (Reynard et al., 1961) and FDP acts as a positive modifier of the enzyme, both these effects having been implicated

in the control of glycolysis and gluconeogenesis (Weber *et al.*, 1966a). There has also been a suggestion that PK may be under endocrinological control, as diethylstilbestrol, (and to a lesser extent certain steroid hormones) effects a change in the viscosity and electrophoretic properties of PK in solution (Kimberg and Yielding, 1962).

Specificity.

PEP is the only known phosphoryl donor in the PK reaction according to Woods *et al.*, (1970) who synthesised seven homologues of this substrate and tested their activity as substrates or inhibitors. This selectivity is marked, as only phosphoenol-α-oxobutyrate and phosphoenol-α-oxovalerate had the capacity to inhibit the reaction with PEP as substrate.

The specificity of the enzyme for the nucleotide coenzyme is less marked. Early reports of phosphoryl donation to GDP, IDP, UDP and CDP (Strominger & Lowry, 1955; Tietz & Ochoa, 1958) were questioned by Davidson (1959) on the basis of the presence of nucleoside diphospho-kinase in the experimental system, which resulted in a transfer of phosphoryl groups from adenosine nucleotides. However, a closer examination of this problem by Plowman and Krall (1965) confirmed the broad specificity and the predilection of the enzyme for the purine nucleotides.

Equilibrium and potential reversibility.

The first demonstration of PEP synthesis from ATP and pyruvate by Lardy and Ziegler (1945), refuted the previously held concept that PK catalysed an essentially irreversible reaction between PEP and ADP. The K_{app} of the reaction is dependent upon the Mg^{2+} ion concentration (McQuate and Utter, 1959), presumably because of the higher equilibrium constant for the binding of Mg^{2+} to ATP than that for the binding of Mg^{2+} to either ADP or PEP. Under physiological conditions, however, the K_{app} appears to be of the order of $6\text{-}7 \times 10^{3}$, and the equilibrium thus lies very much in favour of pyruvate. The apparent $-\Delta F$ for the

reaction is approximately 4700 cal., (McQuate & Utter, 1959), but despite this and the unfavourable K_{app} for PEP synthesis, the ratio of the V_{max} values in the reverse to the forward reaction is only approx. 1/200 at pH 7.4. However, the K_s value for pyruvate estimated by these same authors is 10^{-2}M, and thus *in vivo* the velocity will not approach the V_{max} value.

These facts combine to form a physiologically insurmountable block against the participation of PK in the reversal of glycolysis, and thus we are dealing with a virtually unidirectional process. No evidence has been presented for the presence in *T.rhodesiense* of gluconeogenesis or of the now generally accepted mechanism by which PK is bypassed in gluconeogenesis in higher organisms, viz. by the combination of pyruvate carboxylase, and PEP carboxy-kinase (Utter, Keech & Scrutton 1964). From an experimental viewpoint, the enzymic section of this work has therefore been limited to the utilisation of PEP by the enzyme.

Isoenzymes.

In the absence of a control of PK in the organs of gluconeogenesis, PEP regenerated for gluconeogenesis would be converted into pyruvate. Two means by which this control may be exerted exist in the case of the mammalian liver enzyme. Firstly, the level of hepatic PK is under dietary and hormonal control (Tanaka *et al.*, 1965); in a primarily glycolytic tissue such as muscle, these factors do not affect the enzyme. Secondly, the liver isoenzymes are subject to allosteric control by intermediary metabolites.

Zone electrophoresis of crude muscle and liver extracts by Tanaka *et al.*, (1965), demonstrated one band of activity from the muscle, and

at least four bands from the liver extract. Only one of the four liver isoenzymes was antigenic when immunological assays were carried out with anti-muscle PK serum, and this isoenzyme was named type M. The other three isoenzymes (L_1 L_2 and L_3) were not precipitated by this antibody. Of these three enzymes, only type L_1 has been investigated further, and has been shown to differ from muscle type M in the response to ammonium sulphate precipitation, DEAE-cellulose elution, and inhibition by pCMB, as well as in crystal form and molecular weight (Tanaka et al., 1967[a]).

A danger inherent in the classification of PKs from other tissues as type L or type M, on the basis of immunological or electrophoretic properties, is the lack of correspondance between these properties of the enzymes. Two isoenzymes exist in mammalian kidney, electrophoretically classified as types L and M, but both are precipitated and inhibited by anti-M serum (Tanaka et al., 1965, 1967[a,b]). The only other known source of a type L isoenzyme is the erythrocyte, but whereas the level of the liver L type PK is subject to dietary and hormonal control, the level of the erythrocyte enzyme is independent of these factors. In this respect, then, the erythrocyte enzyme more closely resembles the muscle and liver type M isoenzymes.

The existence of the two isoenzyme forms in the hepatic cell has led Tanaka et al., to postulate the presence of two glycolytic sequences. The diet-, hormone-, and effector-independent type M, in combination with hexokinase (Walker et al., 1963), constitutes, with the rest of the glycolytic enzymes, the basal pathway. When the balance between glycolysis and gluconeogenesis is of special significance, the nutritional and hormonal state of the organism effects changes in the L type PK / glucokinase (Sols & Marco, 1970) pathway, thus altering the net glycolytic flux.

2) KINETIC PROPERTIES.

PK from rabbit muscle conforms to classical Michaelis-Menten kinetics, in so far as both substrates exhibit hyperbolic velocity *versus* substrate concentration curves. (Kachmar & Boyer, 1953; McQuate and Utter, 1959; Boyer, 1962; Reynard *et al.*, 1961). This property is reputedly shared by the type M isoenzyme of liver (Tanaka *et al.*, 1967[a]), the leucocyte enzyme (Campos *et al.*, 1965) and the type L erythrocyte PK (Ibsen *et al.*, 1968). The PKs from yeast (Haeckel *et al.*, 1968) and adipose tissue (Pogson, 1968), and the type L liver isoenzyme, differ in giving 2nd order Lineweaver-Burk plots with varying PEP concentration. The 1st order kinetic pattern of the nucleotide acceptor is in all cases similar to that of the muscle enzyme.

Quantitative estimates of K_M and S_{50} values for the PK of different tissues vary greatly, depending on the conditions used both for extraction of the enzyme, and for assay. Further complications arise from the use of crude tissue extracts, where kinetically different isoenzymes are present (Llorente *et al.*, 1970). Reported K_M PEP values for the erythrocyte enzyme, for example, range from 1.5×10^{-3} M-PEP to 4.6×10^{-5} M-PEP (Campos *et al.*, 1965; Wiesmann *et al.*, 1965; Boivin & Galand, 1968; Ibsen *et al.*, 1968), and whereas only one electrophoretic band of PK appears under the experimental conditions of Tanaka *et al* (1967[a]), there is evidence for the presence of isoenzymic forms of this enzyme (Ibsen *et al.*, 1971; Rudiger *et al.*, 1968). K_M ADP values reported for this enzyme lie in the range $1\text{-}4 \times 10^{-4}$ M-ADP (Wiesmann *et al.*, 1965; Prager and Whigham, 1967; Campos *et al.*, 1965). The greater reproducibility of this parameter may reflect the relative insensitivity of the K_M ADP to modification by intermediary metabolites which may contaminate the enzymic preparations.

The kinetic constants defining the binding of ADP and PEP to PK are probably mutually independent. In the cases of the liver L type and M type enzymes (Tanaka et al., 1967a), the leucocyte enzyme (Campos et al., 1965) the muscle enzyme (Boyer, 1962; Reynard et al., 1961), and the erythrocyte enzyme (Ibsen et al., 1968), the K_M value for each substrate was found to be unaffected by the concentration of the other substrate. However, differences have been found, by Campos et al., (1965) for the erythrocyte enzyme, and by McQuate and Utter (1959) for muscle PK.

Further definition of the K_M and S_{50} values obtained for various PKs would be of little worth at this point, as the reported experimental assay conditions vary drastically with respect to temperature, cation concentration, and degree of enzyme purification.

Regulation of PK by metabolic activators and inhibitors.

As a consequence of the high PK activity in gluconeogenic tissues, some mechanism has been assumed to operate by which PK may be inhibited in the gluconeogenic state and activated when a high rate of glycolysis is required. Hepatic PK is stimulated by FDP (Taylor & Bailey, 1967) and inhibited by NADH (Weber et al., 1965), ATP (Weber et al., 1966a), octanoic acid (Weber et al., 1966b) and Cu^{2+} ions (Carminatti et al., 1968). All these factors have been postulated as exerting a regulatory function in vivo.

i. NADH

The concentration of NADH required for the inhibition of hepatic PK, is much higher than that found under in vivo conditions of gluconeogenesis. However, the extent of this non-competitive inhibition was

shown to be dependent upon the preincubation time of the enzyme with the inhibitor (Weber et al., 1965, 1966a) and a role for this coenzyme in facilitating gluconeogenesis cannot be totally excluded.

ii. ATP

The effect of ATP on PK appears to be dependent upon the enzyme source, and is still a matter of some controversy. Muscle PK has been repeatedly confirmed as being ATP sensitive (Meyerhof & Oesper, 1949; Reynard et al., 1961; Mildvan & Cohn, 1966). Tanaka et al., (1967b) and Reynard et al., (1961) attributed this inhibition to a competitive effect for the enzyme active site, but McQuate & Utter (1959) were of the opinion that Mg^{2+} chelation by the nucleotide, thereby decreasing the effective MgADP concentration, was the basis of the inhibition. Wood (1968), investigated these effects under various conditions and showed that the addition of excess Mg^{2+} ion totally eliminated any inhibition by ATP in the muscle enzyme system.

However, the liver enzyme is inhibited by ATP under conditions where all nucleotide species present are saturated with Mg^{2+}, (Weber et al., 1966a), although the inhibition is potentiated at limiting Mg^{2+} levels, due to the chelation effect. The significance of this inhibition in vivo may therefore be greater than that of NADH. The K_i for ATP in the hepatic enzyme system of Weber et al., (1966a) is 8.0×10^{-5} M-ATP, compared to the in vivo concentration quoted of 2 mM-ATP. ATP also shows cooperative interactions with hepatic PK at acid pH values, indicating rather more than a simple product inhibition.

A rise in the intracellular ATP concentration could therefore act as a negative feed-back control on PK hence limiting its own production in conditions of low energy requirement. Any fluctuations in the

concentration of ATP may therefore alter the glycolytic/gluconeogenic balance, and small variations in pH within the physiological range may also markedly alter the effect of this inhibitor (Rozengurt et al., 1969). It therefore appeared possible that ATP may control the rate of trypanosomal glycolysis.

iii. Free fatty acids

Since the observations by Krebs et al., (1965) and Hanes et al., (1965) that free fatty acids (FFA) stimulate renal and hepatic gluconeogenesis respectively, many key enzymes of the catabolic pathways of glucose metabolism have been shown to be inhibited by FFA, viz. PK, hexokinase, glucokinase and phosphofructokinase (Weber et al., 1966a,b). Glycolytic activity is therefore severely depressed by elevated concentrations of both FFA and their coenzyme A derivatives, with a concomitant increase in the gluconeogenic rate. The effects of these inhibitors were not, however, investigated on the trypanosomal enzyme, on the grounds of the non-specificity of the FFAs (fumarase and isocitrate dehydrogenase are also inhibited) and the fundamental lack of knowledge in the field of trypanosomal lipid metabolism.

iv. FDP

The effects of FDP on the kinetics of PK have been investigated on the enzyme from various sources, including liver (Hess & Haeckel, 1967), adipose tissue (Pogson, 1968) yeast (Hess et al., 1966; Hunsley & Suelter, 1969) and E.coli (Maeba & Sanwal, 1968a,b) and again the details of the activation differ from source to source.

With increasing concentrations of FDP, a progressive increase in PK activity is observed, although the $\overline{A}_{50}$ values for this activator

range from 3.8×10^{-6} M for the liver L enzyme (Weber et al., 1966a) to 1.5×10^{-4} M for the yeast enzyme (Hess et al., 1966). Quantitative discrepancies are again noted in this respect, as Llorente et al., (1970) quote 5×10^{-7} M-FDP as producing "nearly full activation" in liver. However the FDP effect is known to depend upon PEP concentration and also the last figure comes from a source which, despite the publications of the last five years on isoenzymic forms of liver PK, uses a crude extract of liver as an enzyme source. The ratio of L to M type PK in their enzyme preparation is therefore dependent upon the dietary history of their experimental animals, and interpretation of their quantitative results becomes extremely hazardous.

The common qualitative effect of FDP on PK is to alter the shape of the PEP saturation curve from a sigmoid to a hyperbola, increasing the affinity of the enzyme for this substrate. The rat muscle enzyme which displays hyperbolic saturation characteristics to PEP in the absence of FDP, shows virtually no response to this modifier (Llorente et al., 1970), a property shared by the enzyme from eight other tissues tested by these authors. There remains some confusion as to the position of the adipose tissue PK in this respect; Llorente et al., (1970) report a negligible effect of FDP on their enzyme preparation, whereas Pogson (1968) demonstrated a positive response to this modifier. The results of the last author, however, again indicate how pretreatment of the experimental material may alter the kinetic picture. Extraction of adipose tissue with a medium containing EDTA produces a form of PK designated PyK-A, in which form the enzyme is FDP-activated and shows cooperative kinetics towards PEP, Mg^{2+} ions and K^{+} ions. In the absence of EDTA, PyK-B is obtained, which shows normal Michaelis-Menten kinetics with PEP and metal ions, and is FDP insensitive. The dependence of the sigmoidal kinetics on the extraction procedure has

been confirmed (Llorente et al., 1970), and it was further demonstrated that preincubation conditions, temperature, and the age of the experimental animal affect the enzyme characteristics. The latest work of these authors (Marco et al., 1971) would appear to confirm the hypothesis of Pogson (1968) in that adipose tissue PK is FDP-sensitive in vivo.

The yeast enzyme displays cooperative interactions with K^+ ions, the sigmoid nature of the response to increasing concentrations of this cation being abolished by FDP (Hess & Haeckel, 1967). As a comparison, the cooperative effects of Mg^{2+} and K^+ in the adipose tissue system are unaffected by FDP. Hess and Haeckel also found that the V_{max} of the yeast enzyme was altered by FDP when K^+ was the activating monovalent cation, but not when NH_4^+ was used.

No report has yet been published in which a comprehensive investigation of the factors which affect FDP activation is made, namely

1) pH (Taylor & Bailey, 1967).
2) Temperature (Llorente et al., 1970).
3) PEP concentration (Taylor & Bailey, 1967).
4) Cation specificity (Hess & Haeckel, 1967).
5) The presence of FDPase in the enzyme preparation.
6) Extraction procedure (Pogson, 1968).

The effects of pH and temperature are liable to be of minimal physiological significance, and a standard cation concentration has been maintained throughout this work.

Taylor and Bailey (1967) showed that whereas hepatic L type PK was 30% activated at pH 6.0 by 5×10^{-4} M-FDP, over 20-fold activation occurred at pH 8.0. Unfortunately it is impossible to conclude from the data presented whether this simply reflects a protective effect by FDP in alkaline solution. Desensitisation of this enzyme to FDP has

been reported, by storage at 0-2°C (Llorente et al., 1970) or by elevating the PEP assay concentration to 5mM (Taylor & Bailey, 1967). Interestingly, the effect of cold storage is accompanied by an abolition of the homotropic cooperative effects of PEP. These effects may account for the difficulties encountered in obtaining reproducible results for the allosteric behaviour of this enzyme.

Finally, one anomaly is shown in the case of the PK from E.coli (Maeba & Sanwal, 1968a,b), which whilst activated by FDP, is so in such a way that the sigmoidal response to PEP concentration is retained.

v. Cu^{2+} ions

The postulated control of liver L type PK by reciprocal Cu^{2+} inhibition and FDP activation (Carminatti et al., 1968) is of doubtful in vivo significance. The non-competitive nature of the inhibition would suggest that the effects of Cu^{2+} on PK do not have a role in gluconeogenic control. Similarly no significance can at present be ascribed to the reported inhibition of liver PK by Zn^{2+} ions (Solvonuk & Collier, 1955) and the interesting nucleotide specificity of Cu^{2+} ion inhibition (Carminatti et al., 1968).

3) MODELS TO DESCRIBE THE ALLOSTERIC PROPERTIES OF PK

Extensive information about the interaction of PK with the above effectors has been obtained, especially for the liver L type enzyme, and it is therefore possible to propose an allosteric model along the lines of the Monod hypothesis (Monod et al., 1965). It must be noted, however, that whereas the model proposed below acts as a valuable working hypothesis, it is based solely on kinetic data, which is rarely sufficient to distinguish between alternative models describing interaction between

enzymic sub-units and forms. This basic inadequacy of a purely kinetic investigation stems from the major assumption of the R-T classification system of Monod et al., (1965), that molecular symmetry is at all times conserved. This premise cannot be tested kinetically. However, as the relatively simple model on the Monod hypothesis fits the available data, it serves as a summation of the observed modifier and substrate effects on PK.

FDP, ATP and PEP all exhibit under certain conditions, a homotropic cooperative interaction with liver L type PK, demonstrable by the sigmoidal velocity - effector concentration relationships. The homotropic interaction of PK with PEP is abolished by FDP, and the activation of the enzyme by the latter metabolite is eliminated by a high concentration of PEP. Both these modifiers may then be said to combine preferentially with the enzyme in the T state inducing a conformational change to the catalytic R state. Reduction of the pH to a value less than 7.0, eliminates the activating potential of FDP, and transforms the sigmoidal PEP kinetics to a Michaelis-Menten type hyperbola. Hence this treatment of the enzyme also favours the R state.

The effects of ATP mirror these transformation as follows. At acid pH values, ATP interacts cooperatively with L type PK, even at low concentrations of PEP. At pH 7.5, however, the ATP inhibition curve is hyperbolic, unless FDP is present, or a high concentration of PEP is used. Thus the effects of pH change coincide with the modifier effects of FDP and ATP as follows:

$$PK_R \underset{FDP, PEP, H^+}{\overset{ATP \quad OH^-}{\rightleftharpoons}} PK_T$$

The assumptions inherent in this model, 1) that two interconvertible forms of the enzyme exist and 2) that the nature of the substrate saturation curve reflects, in the presence or absence of modifying agents, changes in the distribution of these forms, are in this case probably justified. Recent work by Llorente et al., (1970) indicates that L type PK may be desensitised to FDP activation, ATP inhibition, and PEP homotropic cooperativity, by cold preincubation. This marked desensitisation strongly supports the view that this PK is typical of the allosteric enzymes, and in this respect the enzymes from rabbit (Kayne [& Suelter] 1968) and Alaskan king crab muscles (Somero, 1969) have also been shown to undergo a temperature dependent configuration transition. Unfortunately, as previously stated, the rabbit muscle enzyme portrays no allosteric properties with reference to PEP and FDP, having a hyperbolic response to PEP concentration, and being virtually unaffected by FDP (Taylor & Bailey, 1967). In these respects, assuming that these non-regulatory properties are not artefacts of purification, the rabbit muscle enzyme resembles the PK_R state of the equilibrium postulated for the L type enzyme. If one accepts the evidence of Wood (1968) that muscle PK is ATP insensitive, this enzyme behaves as a "fixed" type of the PK_R mentioned above.

This simple model however does not account for all the data presented to date, especially that of Bailey et al., (1968) who investigated the extremely complex kinetic changes occurring on preincubation of the liver L type enzyme. Without preincubation, the atypical shape of the activity/substrate-concentration curve necessitated the postulate that the enzyme as prepared consisted of a mixture of two PK's, named L_A and L_B. Type L_A is akin to the M.type enzyme in exhibiting a Michaelis-Menten hyperbolic response to substrate concentration and in

being insensitive to FDP. This form is converted into L_B on preincubation at 25°C, producing a conformation which shows a cooperative response to PEP and is FDP sensitive. The effect noted by Carminatti et al., (1968), that FDP effectiveness is markedly enhanced by preincubation at 37°C, confirms these observations. Although Bailey et al., (1968) present some evidence to show that their L_A is not identical with the M type enzyme of Tanaka et al., (1965), they ignore the possible mixture of types L_1 L_2 L_3 (nomenclature of Tanaka et al., 1967) which their abbreviated purification procedure may produce. Isoenzymes L_2 and L_3 were named purely on the bases of their neutralisation with anti-L antibody and their insensitivity to anti-M antibody. The rigorous purification procedure of Tanaka et al., (1967) produced a crystalline, ultracentrifugally pure L_1 type PK. No kinetic analyses have been reported on types L_2 and L_3; one of these isoenzymes could conceivable obtain to hyperbolic kinetics, and could be identical with the L_A enzyme of Bailey et al.

The potentiation of sigmoidicity with respect to PEP, and of FDP sensitivity, on preincubation at 25°C is thus the reverse of the cold desensitisation observed by Llorente et al. (1970), and the temperature dependent conversion appears to be repeatable many times.

Two potential amended models may now be built for the liver L type PK.

$$L_B = T \underset{\text{ATP, } OH^-}{\overset{\text{PEP, FDP, } H^+}{\rightleftharpoons}} R = L_A$$

0°C 25°C

$$L_B(T) \underset{\text{ATP, } OH^-}{\overset{\text{FDP, PEP, } H^+}{\rightleftharpoons}} L_B(R)$$

L_A: 0°C ⇅ 25°C

L_B

At present, insufficient evidence is available to decide whether the temperature sensitivity is a property of the previously described R-T equilibrium, or whether the preincubation at low temperatures shifts the equilibrium to yet another possible enzymic configuration i.e. whether or not the L_A type enzyme of Bailey and Stirpe is identical with the R state.

CHAPTER 3

MATERIALS AND EXPERIMENTAL PROCEDURES

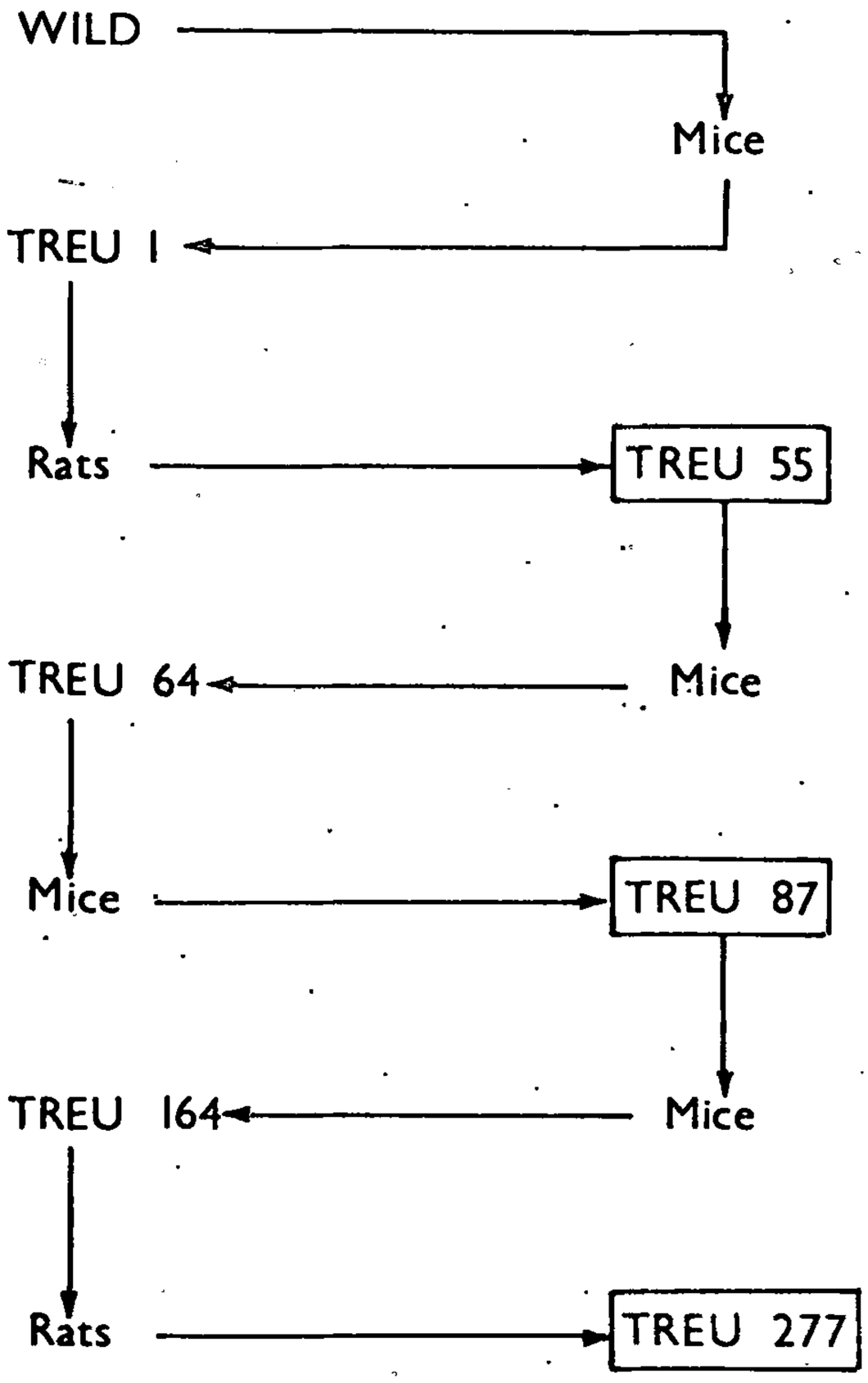


Fig. 2. The derivation of *T.brucei* TREU 277.
The rodent hosts used for passage are as indicated.
The number of passages is unknown.

1) BIOLOGICAL METHODS

Strain maintenance and characteristics.

In the course of this work, several strains of T.rhodesiense and T.brucei were used, the strains varying in their degree of pleomorphism, and in their history, as follows.

The Liverpool strain of T.rhodesiense, ("L" strain), was isolated in 1923 from an infected human patient, and has been maintained subsequently by syringe-passage in rodent hosts. This strain is completely monomorphic, producing a rapidly fulminating infection in rats, which results in the death of the host animals 3-4 days after inoculation.

T.brucei TREU 277 strain (Trypanosomiasis Research, Edinburgh University) originally showed a pleomorphic type of infection, with the second peak of the relapsing parasitaemia resulting in death of the host 9-12 days after infection. On storage at -70°C this strain lost its pleomorphic character over a period of 1-2 years and now yields an infective pattern similar to that of the "L" strain. Figure 2. shows the derivation of TREU 277 and the following two strains, since the original isolation of the organisms. The number of passages to which these strains have been subjected is uncertain.

In the search for a truly pleomorphic strain of trypanosome TREU 55 and TREU 87 T.brucei strains were investigated, to determine whether they would produce a higher yield of short stumpy forms than the TREU 277 strain. Despite the fact that TREU 55 and TREU 87 had been subjected to fewer passages than the final TREU 277 strain, neither of these infections possessed an increased pleomorphic character.

EATRO 165a (East African Trypanosomiasis Research Organisation) is a strain of T.rhodesiense whose history since isolation is again uncertain. This infection behaved in an identical manner to TREU 277,

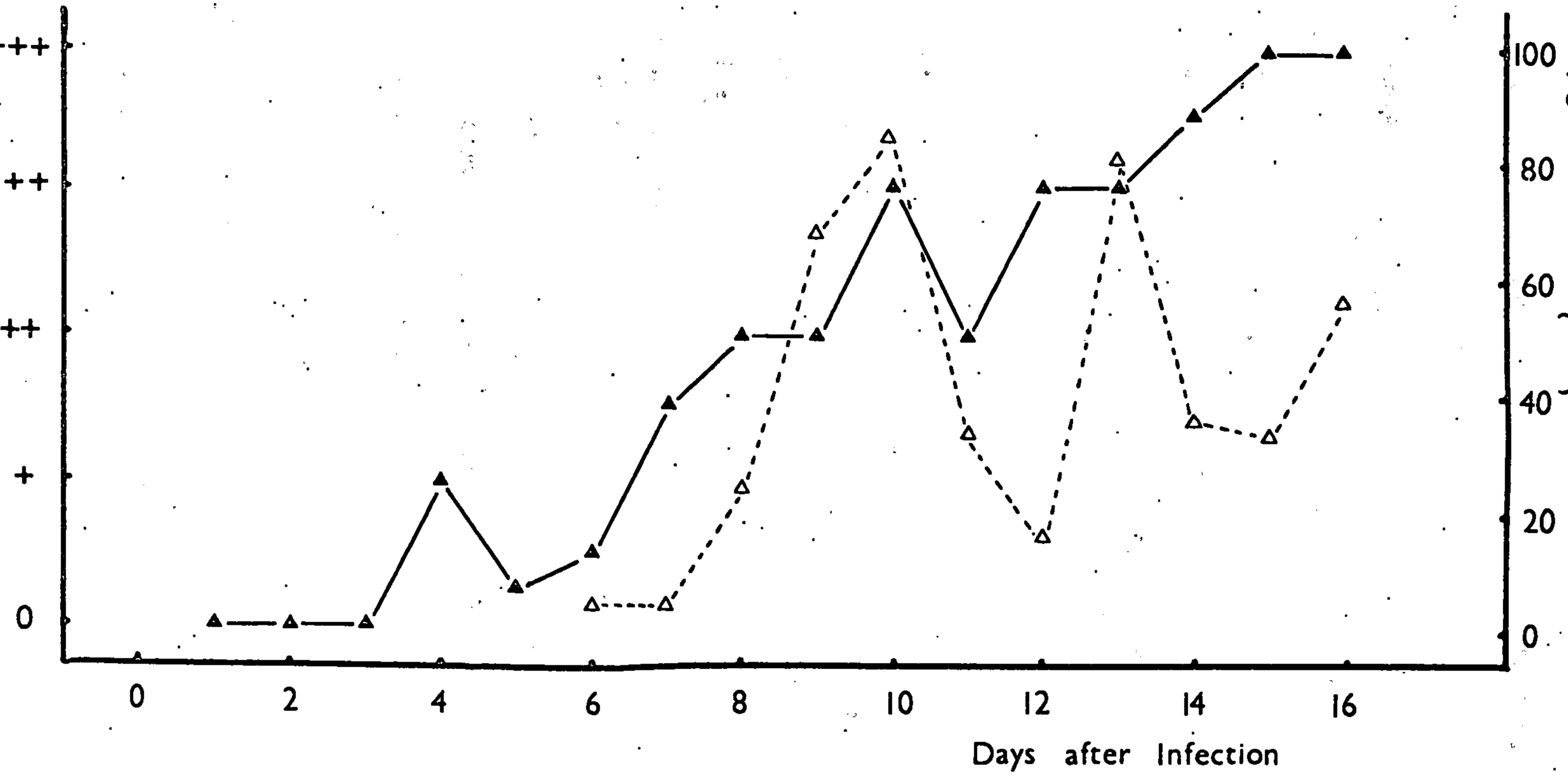


Figure 3. The relationship between the height of parasitaemia and the pleomorphic composition of T. rhodesiense EATRO 173. Daily tail-blood smears were taken from the rat and stained with Leishmans stain. The height of the parasitaemia and the % thick (SS) forms were estimated as described in the text.

undergoing a slow change in character on storage at -70°C. After a period of approximately 5 months, the yields of SS type organisms at any stage of the parasitaemia had markedly decreased, and the infection became gradually more virulent in character. When first obtained, death of the host occurred after about 13-18 days on the third peak of the parasitaemia. After storage for 5 months, more than two phases of the relapsing infection were rarely obtained and some rats succumbed on the first parasitaemic peak after only 5-6 days. However, in the early stages of storage, this strain was used for some of the metabolic studies reported in this work.

T.rhodesiense EATRO 173

This strain was isolated from a human patient in 1962. Unfortunately no record exists of its subsequent history, but it is known that it has been maintained in the frozen state for the majority of the last eight years, with occasional passage through rodent hosts.

This strain showed a satisfactory pleomorphism and has been used throughout the majority of this work. The infection is still of a relapsing nature, although some of the biochemical properties, e.g. the RQ values, have altered slightly since these studies were initiated. The percentage of short stumpy forms present at the various stages of the parasitaemia is now rather lower than the yields obtained originally but infections of 70-80% SS forms are still obtainable.

Characteristics of T.rhodesiense EATRO 173 and identification of SS forms.

The levels of parasitaemia and the percentage of the trypanosomes in the SS form at different times after infection are shown in Figure 3. Parasitaemias were estimated from microscopic inspection of a wet blood film obtained from the tail of a rat, using the following system:

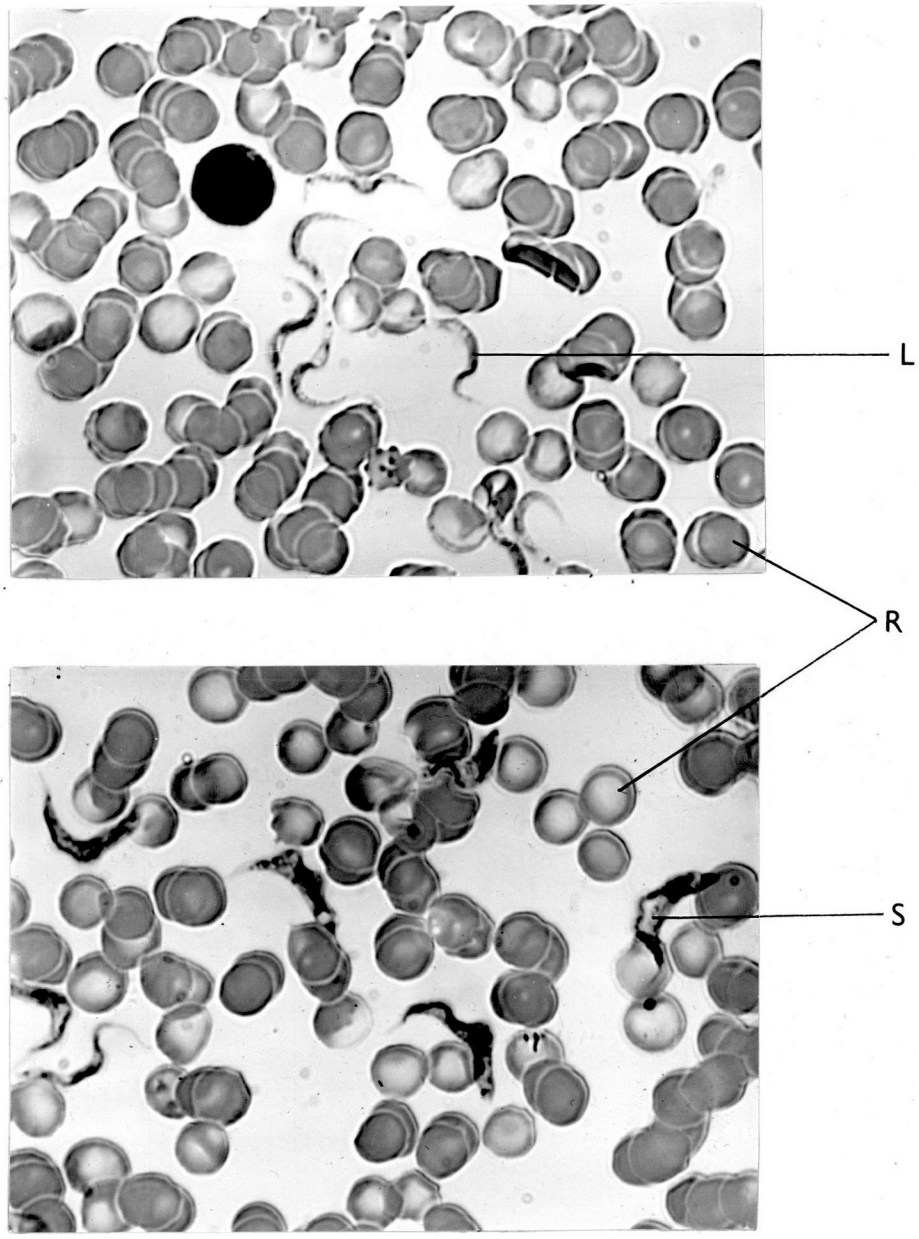


Plate 1. The morphological appearance of the SS and LS forms of T. rhodesiense EATRO 173.

Stained blood smears taken from the relapse (above) and the remission (below) phases of the parasitaemia. L = long slender (LS) form. S = short stumpy (SS) form. R = erythrocyte.

Magnification = 1200x.

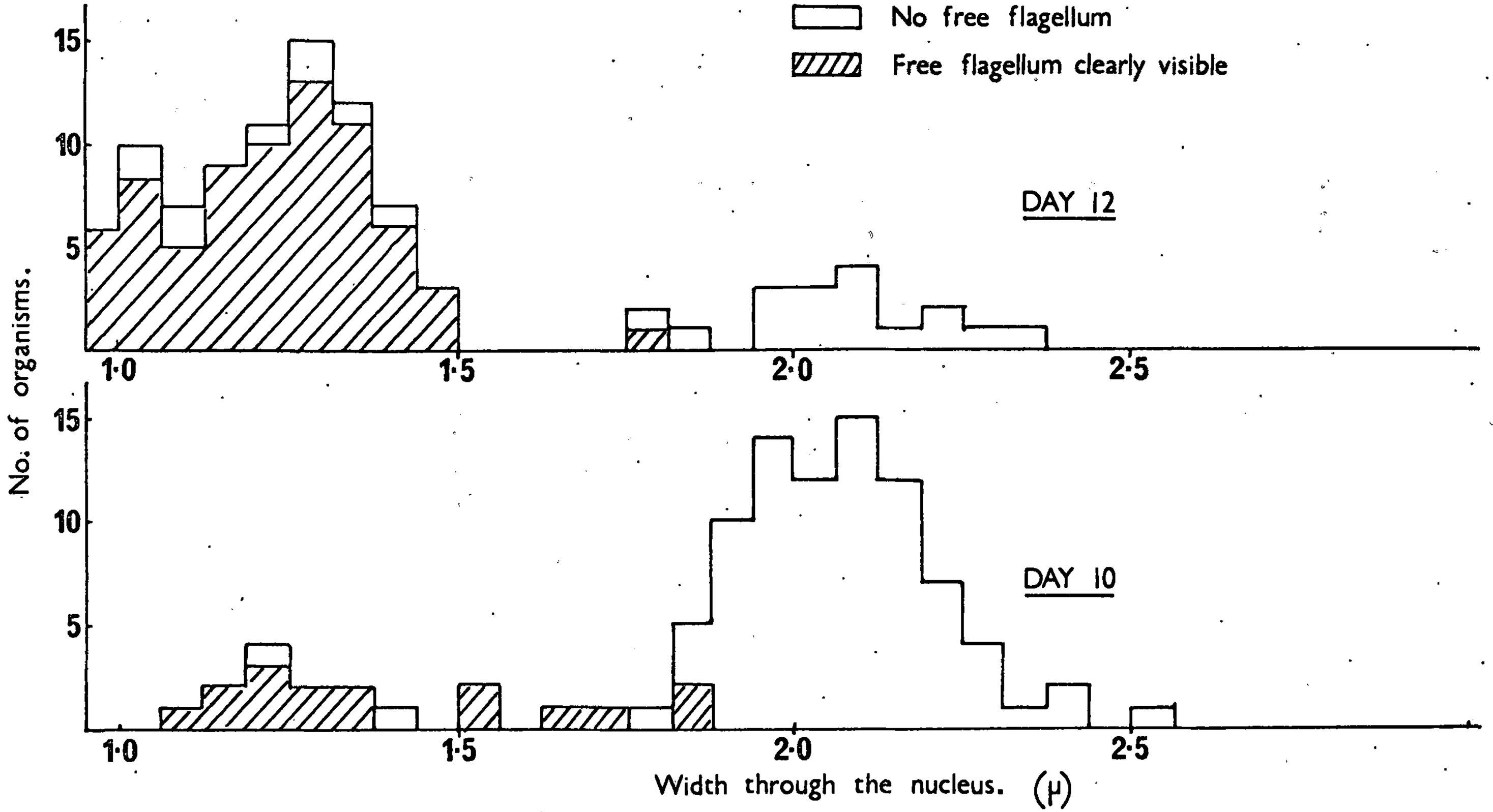


Fig. 4. The relationship between nuclear width and the presence of a free flagellum in T. rhodesiense EATRO 173. Tail-blood smears were obtained from an infected rat 10 and 12 days after inoculation and stained with Leishmans stain. Nuclear width was measured as described in the text.

0 : No trypanosomes visible in 20 fields.

$\pm$: 1 trypanosome every 3-4 fields or fewer.

+ : Approx. 1 trypanosome per field.

++ : 9-10 trypanosomes per field; easily countable.

+++ : Difficult to estimate the trypanosome count/field.

++++ : Very heavy infection only obtained in the 6-12 hour period prior to death.

The SS to LS ratio of the trypanosomal forms was again estimated by microscopic inspection, but in this case a dried blood smear, stained. with Leishmans stain was used. The characteristics used to define a SS form under these conditions were ; lack of a free flagellum, greatly increased width through the nucleus and slightly reduced length. Some organisms inevitably did not fit all these requirements and the deciding factor for classification as SS or LS in these cases was taken as the absence or presence of a free flagellum.

This subjective estimation of the forms of the trypanosome present in a stained smear, was checked on representative samples such as those shown in Figure 4. These data show the measured width through the nucleus of the organisms together with the presence or absence of a free flagellum. It appears, then, that at different stages of the infection a preponderance of the SS or LS forms is found. The correlation of these measurements with the visual estimation of the percentage of short stumpy forms present in a blood smear, showed that the two methods gave comparable results (Fig.5). As a routine method, the simple microscopic examination and subjective estimation on a stained smear was utilised to gauge the type of infection present in the experimental animals. Photographs of two stained samples, pertaining to different stages of the infection, may be seen in Plate 1.

From seven days after infection, tail blood smears from batches of rats were inspected daily, and those animals which showed a combination

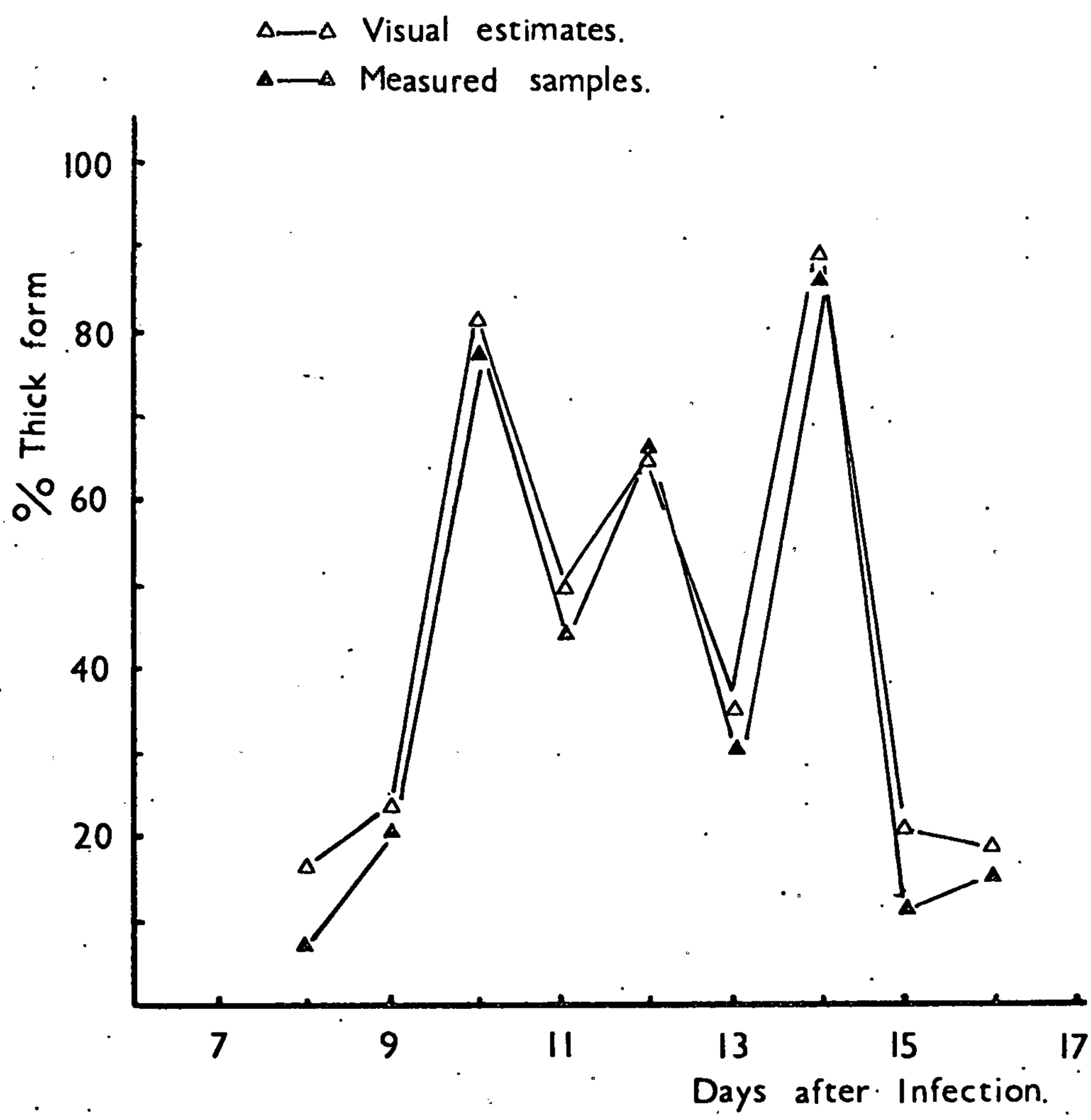


Fig. 5. Comparison of subjective and objective technigues in defining the pleomorphic composition of a trypanosome population. Daily tail-blood smears were taken from an infected rat after the onset of the parasitaemia. The nuclear width of 200 organisms *per diem* was measured on stained smears and those cells having a width of greater than 1.8μ were classified as SS. Visual estimation of the composition of the samples was carried out on unstained smears using the morphological characteristics described in the text.

of a reasonably high parasitaemia with a high percentage of the SS forms were utilised for experimentation.

Staining method.

A drop of infected blood was taken from the tail of the rat onto a microscope slide, spread into a thin film, and stained with Leishman's stain (predissolved, BDH Chemicals, Ltd., Poole). Stained smears were stable for at least a week before the stain faded and the slide became unusable. Where a permanent record of the infection was required, to facilitate measurement at a later date the smears were mounted in neutral XAM obtained from G. Gurrs Ltd. London.

A variation of the Romanowsky type eosin-methylene blue stains, namely Giemsa's stain, was tried in an attempt to produce a clearer definition of the trypanosome outline, especially of the flagellum. However, Leishman's stain gave the most consistent results and was adhered to for routine examination.

Method of infection.

The monomorphic strains were maintained by continual syringe passage. Infected tail blood from a parasitized animal was diluted with three times the volume of citrated saline (0.9% $^{W}/_{v}$ NaCl containing 1.5% ($^{W}/_{v}$) sodium citrate) and 0.5 ml was inoculated intraperitoneally into fresh rats. No restriction was placed on the number of trypanosomes used to infect a host animal in this way, as variation of the infective dose was found only to alter the time of death within the range 3-5 days. Wistar strain white rats were used as host animals throughout this work. No attempt was made to restrict the host animals to a certain weight limit, and male and female rats were used indiscriminately.

Syringe-passage could obviously not be used to maintain the pleomorphic strains of T.rhodesiense, as this treatment is known to lead to loss of pleomorphism (Fairbairn & Culwick, 1947; Ashcroft, 1957). The strains were originally obtained in the form of infected mice or rats and the following method was found to be suitable for the maintenance of the pleomorphic forms in the frozen state.

Three mice were infected with the organism, and the parasites allowed to increase for 3 days. At this time, the parasitaemia was too low to be detected microscopically. The blood from these animals was used to infect a batch of six mice, which were then checked daily until the blood count was of the order of + to ++ on the first peak of parasitaemia. (This process of "blind passage" had the effect of minimising the circulating antibody level of the blood at the time of storage, as the presence of high levels of anti-trypanosome antibody was thought to damage the organisms on prolonged storage). At this stage, the infected mouse blood was collected from the cavernous venous sinus, followed by cardiac puncture, and taken into heparinised Krebs saline (5 I.U. heparin/ml). The pooled infected blood was made 7.5% (v/v) with glycerol, thoroughly mixed and dispensed into glass capillaries, ID approximately 1 mm., and approximately 10 cms in length. Thus about 0.05 ml of infected blood was contained in each capillary. The ends of the glass capillaries were sealed in a flame and the capillaries immersed in ice-cold methanol. The ampoules were slowly cooled to -70°C in solid carbon dioxide, the process being slowed by the packing of the capillaries in polystyrene to increase the time required for complete cooling. After two days, the ampoules were removed from the solid CO_2 and stored in a Revco freezer at -70°C. For infection of a batch of rats, the contents of two such ampoules were diluted in 4 ml citrate - saline and 0.5 ml was injected intraperitoneally into each animal.

The regression of strains TREU 277 and EATRO 165a is thought to have been caused by the use of rat blood for storage, as opposed to mouse blood. Other workers, however, have reported that rat blood may be used with impunity in this respect (Evans D. personal communication). No other explanation for the loss of pleomorphism by these strains has been found.

Preparation of parasites free from blood elements.

The first requirement for this work was the production of a method which completely eliminated erythrocytes, leucocytes and thrombocytes from the trypanosome preparations. The methods available in the literature were in most cases unreliable, as since the work of Dixon (1966) any method of purification which yields trypanosomes having lactate dehydrogenase activity, must be suspect. The following procedure was, however, found to give reasonable yields of apparently undamaged trypanosomes which lacked any LDH activity, and were therefore assumed to be free of blood cell contamination.

Infected blood was withdrawn from the rat by cardiac puncture and diluted with citrate-saline containing 200 mg% (W/v) glucose at 10 ml per ml of blood. $CaCl_2$ (1ml 0.2M) was added for each 10 ml of citrated blood, with continuous shaking in the presence of glass beads, and the shaking continued until the fibrin clot had separated. After filtration through glass wool the blood was centrifuged for 10 minutes at 1000g and the trypanosome layer removed and resuspended in Krebs-saline glucose (vide infra). Rabbit anti-rat erythrocyte serum was added (1 drop per 10 ml) and the infected saline allowed to stand for 10 minutes at room temperature. The agglutinated red cells were packed by a brief (10 second) spin at 1000g and the trypanosome containing supernatant removed. The remaining clumped erythrocytes were eliminated along with

the leucocyte contaminants by filtration of the material through a no.3 sintered glass funnel previously roughened by immersion in a 1:1 (v/v) mixture of HNO_3 and ethanol. (This process made the sintered disc much more effective in the removal of the white cells.) A further centrifugation at 1000g for 10 min. usually showed a few contaminating red cells in the preparation. By sacrificing the lowest layers of the packed trypanosome mass, completely pure organisms could be collected. Resuspension was normally finally in Krebs saline-glucose unless glucose-free organisms were required, when Krebs saline itself was used. In this latter case, the organisms were viable if kept in ice for no longer than 10 minutes, before disintegration occurred of some trypanosomes.

Preparation of trypanosome lysate.

For the metabolic studies the trypanosomes were washed twice in glucose-free saline, suspended in 8 ml glass distilled water per ml packed cells and homogenised by hand with an ice cold teflon pestle in a glass homogeniser at $0^{o}C$. This material was used directly and will be referred to as the standard water lysed preparation. Several other methods of lysis were used for investigation of trypanosomal succin-oxidase activity, and are detailed under the relevant section of Chapter 4.

Attempted separation of SS and LS forms of pleomorphic T. rhodesiense.

Ideally a separation technique yielding a 100% SS form trypanosome suspension was required, and to this end much effort was expended in attempts to separate the bloodstream forms of the organism by use of density gradient centrifugation. Step, linear, and exponential

gradients of sucrose and of Ficoll (a synthetic sucrose polymer, Pharmacia Ltd.,) in Krebs saline glucose were employed with no success in the separation of the LS and SS trypanosomes.

Trypanosomes could readily be separated from erythrocytes and leucocytes, (on e.g. a three-step sucrose gradient of 9%, 13% and 15% ($^{W}/v$) sucrose, after eight minutes centrifugation at 400g, and 2°C), but the thrombocytes were found to have an identical density to the trypanosomes. In the gradient described above, the trypanosomes and platelets are retained in the 9% sucrose band, the erythrocytes in the 13% sucrose band, and the leucocytes distributed between the 13% and 15% sucrose band.

The attempts to separate the pleomorphic cell types were eventually abandoned, and whole trypanosome preparations used as the source of material. For the metabolic studies detailed in this work, preparations which had a SS : LS cell ratio of 75% plus were routinely used. It may be seen from Figure 3 that parasitaemias having a SS cell count of greater than 80% were rarely obtained, although figures as high as 92% were occasionally found. However, as the infected blood of at least three rats was routinely used per diem, the final trypanosome preparation was never higher than 84% SS nor lower than 78% SS, unless a "thin" preparation was especially required. For this reason, no attempt was made to adjust the metabolic figures obtained to account for the minimal variation in SS-LS cell ratios. Where metabolic differences between the morphological types became apparent (e.g. in the oxidation of α-OG), these differences have been correlated with the percentage of thick forms present in the material.

Preparation of rabbit serum sensitised to rat erythrocytes.

A rabbit was subjected to a course of six injections over a period of three weeks, each dose consisting of 2 ml of a 50% ($^{v}/v$) suspension of washed rat erythrocytes in Krebs saline. The injections were given via a marginal ear vein and when a sample of the animals' serum obtained from the same source showed an adequate antibody titre to normal rat cells, the rabbit was anaesthetised and exsanguinated via the femoral artery.

The blood was allowed to clot at room temperature for 5 hours and the retracted clot removed by centrifugation. The clear serum was dispensed in approximately 2 ml aliquots and stored at $+2^{\circ}C$. The activity of this stabilate was not markedly decreased over the longest period of storage used, of 11 months.

Measurement of gas exchange.

Oxygen utilisation and carbon dioxide evolution were measured in a conventional Warburg apparatus at $37^{\circ}C$ with air as the gas phase. The fluid volume was in all cases 3.0 ml with 0.1 ml of 40% ($^{w}/v$) KOH or 0.1 ml of Krebs saline in the centre wells of the flasks, absorbed on fluted filter paper strips. Temperature equilibration of the flasks was carried out for 10 minutes prior to the start of the experiments, and the measurements were terminated by the addition of 0.5 ml 0.33 M-PCA from the side arm. Single side arm flasks were used unless otherwise stated in the text. For the investigation of the effect of cyanide, centre well mixtures of KOH and KCN and reaction concentrations of cyanide were calculated according to the method of Robbie (1946). The flasks were continually mixed by agitation at 110 rpm through an arc of 26 degrees.

TABLE 1

Effect of supplementation on oxygen utilisation by lysed cells

Standard water-lysed preparations (approx. 1.0 mg N) of T.rhodesiense EATRO 173 were suspended in 1.0 ml saline with the constituents as shown below in a final volume of 3.0 ml. Oxygen uptakes were measured manometrically over a period of 30 minutes in the presence of 25 µmoles glucose. Utilisations expressed on the basis of the fully fortified sample = 100.

Expt.	Vessel contents	Rate of oxygen uptake - %
1	Glucose	4
2	As 1 + ATP (5 µmol)	20
3	As 2 + EDTA (3 µmol)	24
4	As 3 + BSA (10 mg)	40
5	As 4 + Mg^{2+} (20 µmol)	48
6	As 5 + K^{+} (200 µmol)	52
7	As 6 + ADP (5 µmol)	64
8	As 7 + NAD^{+} (5 µmol)	88
9	As 8 + Nicotinamide (25 µmol)	100

Careful washing of the organisms in glucose free saline ensured that no endogenous gas exchange occurred, and occasional checks on the lack of oxygen uptake in the absence of exogenous substrate were carried out.

Experiments using whole organisms necessitated the solution of all substrates and inhibitors in isotonic saline to avoid cell rupture. The use of the standard water-lysed preparation led to problems of fortification to correct for the dilution of coenzymes and intracellular salts occurring on lysis. The minimal supplementation used was based on preliminary tests, some of the results of which are shown in Table 1. Nicotinamide is included to inhibit the activity of NADase, and the two cations are known to be specific requirements for glycolytic kinases. Bovine serum albumin (BSA) stabilises the L.∝GP oxidase system (Grant & Sargent 1960) and markedly increases the time over which the oxygen utilisation rate is linear. Ethylene diamine tetracetate (EDTA), presumably acting in its normal rôle as a trace metal chelating agent, had a similar effect to BSA. The data in this table are for optimal concentrations of the supplements. For all subsequent experiments using the standard water-lysed material, the following buffer system was used at 1.0 ml per flask, the flask fluid volume being standardised to 3.0 ml with H_2O after addition of any other materials:

Standard Warburg buffer:

KCl	0.05 M	titrated to pH 7.4 with 0.1 M-HCl.
$MgSO_4$	0.1 M	
Nicotinamide	8×10^{-3} M	
EDTA	10^{-3} M	
BSA	10 mg ml^{-1}	
Na_2HPO_4	0.02 M	

Where ADP and NAD^+ were added as supplementation, 5 µmole of each coenzyme was added to each flask. Throughout the text, the combination of the standard Warburg buffer and ADP + NAD^+ will be referred to as minimally fortified medium (MFM).

For the estimation of the very low succinoxidase activity, and the investigation of the effects of further supplementation on the ∝-keto acid oxidases, a polarographic system was used. Oxygen uptake was measured at 25°C with a Clark oxygen electrode polarised at -800 mV. The electrode was fitted to a perspex reaction vessel of 3 ml capacity, equipped with a thermostatically regulated water-jacket and connected to a Servoscribe Model RE511 graphic recorder. Ten minutes were allowed for temperature equilibration, and the reactions started by addition of substrate (10 - 100 µl) through an aperture in the perspex chamber.

2) CHEMICAL METHODS.

Quantitative estimation of substrate utilisation and metabolic end products.

At the end of the period of incubation in the Warburg apparatus, the reactions were terminated by addition of 0.5 ml of 0.33 M-PCA from the flask side-arm. Absorption of gaseous CO_2 by the centre-well KOH was complete 20 minutes after acidification, at which time the gas exchange was measured. The flask contents were removed, centrifuged for 10 minutes at 2000g, and the supernatants were separated. A part (2.5 ml) of the supernatants was neutralised by addition of 0.6 M-K_2HPO_4, and the neutralised fractions were stored at +2°C for at least six hours to precipitate the perchlorate as the potassium salt. After centrifugal removal of the precipitate, aliquots of the supernatants were used for

the determination of intermediary metabolites. For studies on carbon balances from glucose, pyruvate or α-OG, sample flasks were treated in an identical fashion, with the addition of perchloric acid from the side-arms immediately after the closure of the taps at the start of the experiments. The analyses from these flasks were used as "T_o" samples, to enable the definition of substrate utilisation and product formation over the same period as the gas exchange.

Estimation of intermediary metabolites.

i. Glucose

Glucose was determined by use of commercially produced "Glucostat" kits (Worthington Biochemical Corporation, Freehold, New J.) in which the hydrogen peroxide produced by glucose oxidase action is used to oxidise a colourless chromogen to a coloured product as follows:

$$\text{glucose} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} H_2O_2 + \text{gluconic acid}$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow[\text{peroxidase}]{} \text{oxidised chromogen} + H_2O$$

The colour absorbing at 400 nm is stable for at least two hours if the enzymic reaction is terminated with 4M-HCl. Standard glucose solutions were taken through the deproteinisation procedure prior to use as standards for the assay. As the glucose oxidase is specific for β-D-glucose, freshly prepared standard solutions were allowed to stand for several hours before use, to allow the mutarotation process to reach equilibrium. Standard curves obtained were linear up to 200 μg glucose/assay; by inspection of the oxygen uptakes obtained manometrically, a rough estimate of the glucose utilisations could be formed, and the aliquots of the deproteinates chosen to contain from 50-150 μg glucose. All estimates were carried out in duplicate.

ii. Pyruvate and phosphoenolpyruvate.

Deproteinised samples (50 µl) were incubated in 0.2 M-K^+ phosphate pH 7.0 (2.0 ml) with 0.45 µmole NADH in a total volume of 2.98 ml. After measurement of the extinction at 340 nm, 20 µl lactate dehydrogenase (approx. 50 I.U.) were added and the reaction mixtures left for 30 minutes at room temperature. The pyruvate concentrations in the experimental aliquots could then be calculated from the decrease in extinction at 340 nm, using the molar extinction coefficient of 6.22×10^3 for NADH. Blank estimations containing no LDH were used to correct for autoxidation of the reduced coenzyme.

Phosphoenolpyruvate could then be estimated by the addition to the final pyruvate estimation solutions, of 20 µl of a solution containing 20 µmoles $MgSO_4$, 5 I.U. of rabbit muscle pyruvate kinase, and 2 µmoles ADP.

The reactions occurring:

$$PEP + ADP \longrightarrow Pyruvate + ATP$$

$$Pyruvate + NADH + H^+ \longrightarrow Lactate + NAD^+$$

enable the PEP concentration to be obtained from the further decrease in absorbance at 340 nm after correction for the dilution factor.

iii. Acetate.

Acetate was estimated by the method of Soodak (1962) utilising the sulphanilamide acetylating enzyme of pigeon liver. In the range 0.025 to 0.20 µmole of acetate, a linear response is obtained to the concentration of acetate present, measured as a disappearance of free sulphanilamide. Estimations were carried out in duplicate, and each run contained four standard acetate solutions, from 0.03 -- 0.20 µmole acetate, prepared via the same deproteinisation method as the flask contents.

iv. Succinate.

The determination of succinate was carried out enzymically, using the succinoxidase of rat liver mitochondria to reduce ferricyanide (Singer et al., 1962). Mitochondria were prepared by the method of Weinbach (1961).

With the low concentrations of succinate produced in the Warburg flasks, direct estimations of the reduction of ferricyanide at 410 nm gave very low spectrophotometric values and hence a low accuracy. A much higher accuracy was obtained by estimation of the ferrocyanide produced, by coupling to a ferric salt to give ferric ferrocyanide (Prussian blue) and estimating this compound at 620 nm.

After incubation of the samples with washed mitochondria (0.1 ml containign 5-10 mg protein) for 40 minutes in the presence of ferricyanide (0.2 ml, 0.01 M), 5.0 ml of ferric reagent (vide infra) were added and the mixture allowed to stand at room temperature. Approximately 2 - 3 hours were required for full development of the blue colour, but linearity of colour versus succinate concentration is obtained after 15 minutes. Ferric reagent was prepared as follows: Recrystallised ferric nitrate (5 gm) was dissolved in 500 ml of double distilled water, mixed thoroughly with 75 ml of "Analar" orthophosphoric acid and diluted to one litre. This reagent normally required filtration before use, as a light precipitate formed gradually on standing. As with all other analytical procedures estimations were carried out in duplicate, and standard succinate samples were taken through the deproteinisation procedure prior to use.

v. Glycerol.

The standard reaction mixture for the spectrophotometric estimation of glycerol was made up as follows, in a final volume of 2.0 ml:

100	µmole	Tris buffer pH 7.5
200	µmole	KCl
20	µmole	$MgSO_4$
2	µmole	EDTA
5	µmole	PEP
5	µmole	ATP
5	I.U.	Pyruvate kinase
50	I.U.	LDH
1	µmole	NADH

A suitable aliquot of the experimental sample containing approximately 0.1 µmole glycerol was added, and the system allowed to come to equilibrium. At this point, most or all of the pyruvate present in the sample will have been converted to lactate with the concomitant utilisation of NADH. Simultaneously any ADP contaminating the ATP, or present in the sample will have been phosphorylated at the expense of PEP, and the pyruvate produced again reduced by the lactate dehydrogenase. NADH was then added to the system in aliquots of 0.01 ml containing 0.05 µmole NADH, until the extinction at 340 nm was stable at between 0.8 and 0.9, and the volume of the assay mixture then diluted to 2.95 ml. This dilution decreased the measured extinction at 340 nm to approximately 0.8 units. Addition at this point of 50 µl of glycerokinase (5 I. units) resulted in the following reactions:

$$\text{Glycerol} + \text{ATP} \longrightarrow \text{Glycerophosphate} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \longrightarrow \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \longrightarrow \text{Lactate} + \text{NAD}^{+}$$

Hence the decrease in extinction of the solution at 340 nm after correction for the dilution factor, enabled the concentration of glycerol present in the sample to be estimated.

vi. ∝-oxoglutarate

Total keto-acids were estimated in the deproteinised samples by the method of Friedemann and Haugen (1943), utilising the extinction band at 500 nm of the 2.4 dinitrophenylhydrazone. The concentration of ∝-OG in a sample is estimable by subtraction of the pyruvate estimate from the total keto-acid figure.

Samples (100 µl) were warmed for 10 minutes at 25°C, mixed with hydrazine reagent (1.0 ml; see below) and allowed to stand for 5 minutes prior to addition of 2.5 M-NaOH. Ten minutes after addition of the alkali, the extinction at 500 nm was measured. Standard curves were prepared both with ∝-OG and with ∝-OG/pyruvate mixtures.

Hydrazine reagent

2,4 dinitrophenylhydrazine (100 mg) is ground in a mortar with small volumes of 2N-HCl and made up to 100 ml in this reagent. This solution was filtered and stored at +2°C.

vii. Citrate.

Citrate was estimated by the spectrophotometric estimation of NADPH formation in the presence of aconitase and iso-citrate dehydrogenase. (Siebert, 1965).

viii. Hexose monophosphate.

Glucose-6-phosphate and fructose-6-phosphate were estimated by the utilisation of NADPH by glucose-6-phosphate dehydrogenase in the presence of phosphoglucose isomerase (Hohorst, 1965).

Estimation of haem pigments.

Spectrophotometric estimation of cytochromes was carried out on a Unicam SP 800 recording spectrophotometer in 1 cm path length quartz cells. The scale length was expanded up to 20-fold by coupling the spectrophotometer to either a Servoscribe R.E. 511 potentiometric slave recorder, or to a Unicam SP 20 recorder. At the maximum range of scale expansion (x20) a scale width of 0 - 0.1 extinction units is obtained on this instrumentation.

The more sensitive chemical estimation involving the production and estimation of pyridine haemochromes was carried out essentially by the method of Falk (1964).

Estimation of protein.

Protein was estimated as follows, after the method of Lowry et al., (1951).

Reagents
- A 2% (W/v) Na_2CO_3 in 0.1 N-NaOH
- B 0.5% (W/v) $CuSO_4 . 5H_2O$
- C 1.0% (W/v) Sodium citrate
- D Folin-Ciocalteau reagent (BDH biochemicals) diluted 1 : 1 with water.

Reagents A, B and C are mixed in the ratio 100 : 1 : 1 by volume, to produce reagent E. The sample (1.0 ml, approx. 0 - 250 µgm protein) is added to reagent E (5.0 ml), thoroughly mixed, and allowed to stand at room temperature for 10 minutes. Reagent D (0.5 ml) is added, the mixture is thoroughly agitated, and after a further 30 minutes at room temperature, the extinction at 660 nm is measured on a SP 800 recording spectrophotometer.

The concentration of protein is estimated by comparison with a standard curve of extinction at 660 nm versus /ug protein, produced by the use of a standard solution of BSA. This standard graph is linear up to 250 /ug protein/assay.

The spectrophotometric determination of protein at 280 nm and 260 nm is that of Warburg and Christian (1942).

Estimation of total nitrogen.

Lyophilised trypanosomes (approx. 20 mg) were digested in a Kjeldahl flask with catalyst (250 mg, $CuSO_4$ / Na_2SeO_4) and conc. H_2SO_4 (2.0 ml). After digestion, the flask contents were diluted to 20.0 ml and 4.0 ml aliquots were steam-distilled on a Markham still into 5 ml ethanolic 1% (^{W}/v) boric acid / mixed indicator (vide infra). The NH_3 content was determined by back titration with standardised approx. 0.01 M-HCl. Ammonium sulphate solutions of known strength were used to calibrate the standard acid.

Mixed indicator.

Methyl red (70 mg) and bromocresol green (40 mg) were dissolved in 50 ml ethanol, and 3 drops of this indicator were used per assay.

Krebs-Ringer Phosphate Saline (Krebs saline).

This isotonic saline has the following constituents:

0.9% (^{W}/v) NaCl	100 parts
1.15%(^{W}/v) KCl	4 parts
0.1-M-phosphate (Na^+) buffer pH 7.4	20 parts
3.82% (^{W}/v) $MgSO_4 \cdot 7H_2O$	1 part

Where Krebs saline glucose is referred to in the text, glucose was dissolved in this saline to a final concentration of 2 mg/ml.

Estimation of protein thiol groups.

The method of Ellman (1959) was used as follows. An aliquot (.02 ml) of Ellmans reagent (39.6 mg 5,5 dithio bis - (2 nitrobenzoic acid) in 10 ml 0.05 M-K_2HPO_4 pH 7.0) was added to a mixture of 0.05 M-K_2HPO_4 pH 8.0 (0.6 ml), resuspended lyophilised trypanosomes (0.5 ml, 3 mg /ml), various concentrations of melarsen oxide, and water, in a final volume of 2.98 ml. After 30 minutes incubation at room temperature, the absorbance of the solutions at 412 nm was measured, and after correction for the blank solutions, this absorbance was taken as proportional to the thiol concentration in solution.

Estimation of inorganic phosphate.

Inorganic phosphate was estimated by the method of Bartlett (1959).

Chemicals.

All chemicals used were of the highest available purity, and were purchased from the following suppliers.

LDH	GDP	NADH	Coenzyme A	MDH
ADP	UDP	FDP	α-oxoglutarate	PK (Rabbit muscle)
ATP	CDP	F6P	Glutathione	G6PDH
PEP	NAD^+	G6P	ICDH	Hexose isomerase
Malic acid	Pyruvic acid	Aspartic acid	Citric acid	

Boeringer & Sohne (London) Ltd., London W.5. U.K.

Dithiothreitol : Koch Light Laboratories, Colnbrook, Bucks, England.

DL-α-glycerophosphate : Sigma Chemical Company

DL-isocitric acid, trisodium salt
L-proline.
Thiamine pyrophosphate
Oxaloacetic acid
α-Lipoic acid
} BDH Biochemicals

BSA Armour Pharmaceutical Co. Ltd. Eastbourne, England.

All other reagents and solvents were "Analar" grade, purchased from BDH biochemicals.

The trivalent and pentavalent arsenicals used in this work were a gift from May & Baker Ltd., and this gift is gratefully acknowledged.

Biogel-A is a spherical agarose gel for filtration and is produced by Calbiochemicals Ltd.

Preparation of the arsenicals for use.

Both melarsen oxide and phenylarsenoxide are insoluble in water. The standard preparative method employed with these drugs was to dissolve them in 40% ($^{W}/_{v}$) KOH soln., dilute to approximately the required volume with water, and titrate to the required pH with 5N - HCl immediately. If these compounds are allowed to stand in alkaline (pH $\geqslant$ 9.0) solution for longer than ten minutes, a cloudy solution is obtained on back-titration to neutral pH values. This may be due to the relative instability of the S-triazine ring in strong alkali.

Immediate titration produces clear solutions, but after approximately 4 hours at room temperature, an opalescence appears. For this reason, no solution older than two hours was used for any purpose.

Assay method for pyruvate kinase.

Assay of PK activity is usually carried out by estimation of the rate of production of pyruvate. Two methods are available for this estimation, a sampling technique in which the pyruvate is measured chemically by estimation of the 2.4 dinitrophenylhydrazone(Kachmar et al., 1953) and an enzymic technique. The latter method, based on the decrease in the 340 nm absorbance of NADH, depends on the coupling of the pyruvate to lactate dehydrogenase producing lactate at the expense of NADH (Bucher & Pfleiderer, 1962). This assay has the advantage of continuously removing pyruvate, eliminating any possible product interference with the enzyme activity.

Alternatively, substrate utilisation may be measured, instead of product formation. The PEP remaining in the assay mixture may be cleaved to produce inorganic orthophosphate, by mercuric salts or alkaline iodine solution (Lohmann & Meyerhof, 1934) but again this method

entails sampling and cannot be used for continuous monitoring of activity. In 1967, Pon & Bondar suggested that the PK reaction may be followed directly by recording the change in optical density of the assay mixture at 230 nm. This method is based on the much larger absorption of PEP than that of pyruvate at this wavelength and provides a direct, sensitive and continuous assay method. Unfortunately, it has two disadvantages. Firstly, pyruvate is not removed from the system and may interfere to produce non-linear rates. And secondly, the sensitivity of most standard spectrophotometers is limited at this wavelength, especially in the presence of high concentrations of protein which necessitates in many cases a base line shift due to the protein absorbing strongly in this range.

In this work, therefore, the analysis of PK activity, for reasons of continuity and removal of product, was carried out by the compound optical assay system of Bücher and Pfleiderer (1962) according to the following equation:

$$\text{PEP} + \text{ADP} + \text{H}^+ \xrightarrow[Mg^{++}\ K^+]{\text{PK}} \text{Pyruvate} + \text{ATP} \xrightarrow[\text{NADH}]{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

All reagents are pipetted in order into a silica cuvette (1 cm light path) and pre-equilibrated in the spectrophotometer for $\geqslant$ 3 minutes at 25°C. The spectrophotometer used was a Unicam model S.P. 800, fitted with a scale expansion unit and coupled to a slave recorder, Servoscribe model RE.211. Earlier experiments were made on the Unicam spectrophotometer model S.P. 500 which did not have a facility for fitting a continuous read-out slave recorder, and in this case, readings were taken manually every 15 seconds.

Full scale sensitivity of 0 - 1.0 extinction units and a chart speed of 600 mm hr^{-1} were used unless otherwise stated. The cell compartment in the SP 800 was maintained at 25°C ± 0.6°C by means of a circulating water bath and pump. The reaction was started by addition of enzyme, again unless otherwise stated, and initial velocities were estimated by tangential measurement to the recorded curve with crude enzyme, or by the gradient of the rate line using purified preparations.

The variation in cation and coenzyme requirements between PK's of various tissues and species necessitated the investigation of the special requirements of the trypanosomal enzyme. The results of the K^+, Mg^{2+} and ADP titrations carried out to obtain optimal conditions will be found in Chapter 5. Theoretically, saturating concentrations of all reactants should have been used for assay of the enzyme, but to avoid wastage of ADP and PEP, the following assay system was utilised for routine analysis.

Reagent.	Volume.	Final Concentration.
0.1 M TEA	1.0 ml	33.3 mM
0.2 M $MgSO_4$	0.1 ml	6.67 mM
2 M KCl	0.1 ml	66.7 mM
4 M NADH	0.1 ml	1.33 mM
LDH	0.05 ml	5 I.U.
0.0125 M ADP	0.1 ml	0.41 mM
0.05 M PEP	0.1 ml	1.67 mM

+ enzyme and water to 3.0 ml.

Nomenclature.

The relationship between the substrate concentration and the initial reaction velocity for an enzyme possessing Michaelis-Menten

kinetics may be described by the two constants V_{max} and K_m. However, many enzymes are now known to show a sigmoid response of initial velocity to increasing substrate concentration and in these cases two parameters are inadequate to describe the enzyme reaction. The substrate concentration required to produce an initial velocity equal to $\frac{1}{2}V_{max}$ in such a system will therefore be designated S_{50} in accordance with the suggestion of Koshland (1966). This parameter will have the dimensions of a K_m value but will be a function of more than one equilibrium constant. The V_{max} in a sigmoid system retains its usual significance and the third parameter "n" (see below) defines the degree of sigmoidicity of the initial velocity versus substrate concentration plot.

An obvious extension of this nomenclature is the use in this work of the terms A_{50}, to designate the concentration of a modifying compound required to give half maximal activation and I_{50} denoting the concentration of inhibitor giving 50% inhibition under a given set of experimental conditions.

Treatment of kinetic data

In all cases, kinetic data were converted to a linear form prior to analysis. Where the reaction order with respect to a substrate or modifier was possibly higher than first order, the plot of

$$\log \frac{v}{V_m - v} \quad \underline{vs} \quad \log S$$

has been used to determine the kinetic constants. This plot, based on the assumptions of Hill (1913) that in the reaction

$$E + nA \rightleftharpoons EA_n$$

there are no intermediate E-A complexes and that the ($EA_n \longrightarrow EA_{n-1}$ + product) step is rate-limiting is based on the kinetic equation

$$\frac{v}{V_{max}} = \frac{1}{1 + (\frac{K}{A})^n}$$

Obviously, as a log/log plot, the Hill plot will be relatively insensitive, and is theoretically non-linear at very high or very low values of v. However, more rigid analysis on the model of Monod et al., (1965) in terms of L (the conformational equilibrium constant) and c (the relative ligand binding capacities of the configurations) is not possible in the present case. Interpretation of kinetic data in terms of these descriptive parameters relies heavily on the regions of activity where v is less than 10% and greater than 90% of the maximum velocity (Koshland et al., 1966). At present, the properties of trypanosome PK in the purified state make analysis in these regions comparatively unreliable.

The data in this work have therefore been analysed in terms of the Hill plot, and the three parameters V_{max}, S_{50} and n have been used to describe the enzyme. Estimates of V_{max} were obtained from double reciprocal plots and used to calculate the ordinate values in the plot of log $\frac{v}{V_m - v}$ vs log (S). The S_{50} values are taken as the intercepts of these plots on the abscissa, and the slopes at this point are taken as the values of n. This analysis does not therefore allow any quantitative significance to be attached to the value of the "interaction coefficient" in terms of the number of substrate binding sites. Values of $n > 1.0$ have therefore been taken only as indicative of the existence of multiple PEP-binding sites whose degree of interaction and co-operativity is dependent upon the presence or absence of a modifier (FDP), and no quantitation of these interactions has been attempted.

All linear data were subjected to regression analysis, the gradient and intercepts being obtained with an Olivetti desk computer, model "Programma-101." My thanks are due to Dr. I.A. Nimmo of the Biochemistry Department, University of Edinburgh, for the preparation of the programs.

CHAPTER 4

EXPERIMENTAL AND RESULTS -

METABOLIC STUDIES

1.) METABOLITE UTILISATION BY T.BRUCEI AND T.RHODESIENSE

Utilisation of extracellular substrates by whole cells

The observation of Vickerman (1962) that the SS forms of T.rhodesiense retain their motility in solutions of α-oxoglutarate (α-OG) whereas the LS forms become immotile, indicated that the pleomorphic types differed in their abilities to utilise this substrate as an energy source. The capacities of a pleomorphic and a monomorphic strain to utilise various substrates, are shown in Table 2.

In the absence of a readily utilisable substrate, the trypanosomes rapidly lost their motility and lysed. This sensitivity of the isolated organisms was noted by Ryley (1962), and casts some doubt on experimental results obtained over a longer incubation period e.g. Grant & Fulton (1957) - 2 hours. The minimal utilisations obtained with all substrates except glucose, glycerol and α-OG with EATRO 173, and glucose and glycerol with TREU 277, are similarly ascribable to cell lysis prior to utilisation, rather than signifying a capacity of the intact organisms to metabolise these compounds.

Inspection of a suspension of the pleomorphic strain supplied with α-OG as substrate, confirmed the preferential survival of the SS forms, the LS forms rapidly becoming immotile and disintegrating. The ability of the pleomorphic strain to oxidase α-OG and the significant carbon dioxide evolution from the utilisation of glucose or glycerol, are the outstanding differences between the strains found on this preliminary survey. Individual estimates of the R.Q. values ranged from 0.48 - 0.62 for the utilisation of glucose by EATRO 173, and from 1.58 - 1.69 for the utilisation of α-OG by the same strain. After storage of the organisms at -70°C for approx. 2 years, the glucose dependent R.Q.

TABLE 2

Oxygen utilisation by whole cells in the presence of various substrates

Whole cells (approx. 0.2 mg N) were suspended in saline with 20 μmoles substrate per flask under the standard manometric conditions described in Chapter 3. Where oxygen utilisation was at a rate greater than 1.0 μl/min/mg N, the exchange over a period of 30 minutes was measured. In all other cases, initial rates were estimated. Pleomorphic composition of EATRO 173 = 78 - 81% SS.

	T. rhodesiense EATRO 173			T.brucei TREU 277		
	O_2 uptake	CO_2 evolution	R.Q.	O_2 uptake	CO_2 evolution	R.Q.
	(μl / min / mg N)			(μl / min/ mg N)		
Glucose	17.7(12)	10.4(7)	0.59	19.1(4)	0.1(4)	0.01
Glycerol	18.4(5)	9.4(2)	0.51	21.3(2)	0.1(2)	0.01
α-Oxoglutarate	8.4(6)	13.9(5)	1.64	0.2(2)	0.1(2)	-
Succinate	0.5(2)	0.5(2)	-	0.2(2)	0.1(2)	-
Fumarate	0.3(2)	0.3(2)	-	0.2(2)	0.1(2)	-
Pyruvate	0.4(2)	0.6(2)	-	0.2(2)	0.1(2)	-
Malaté	0.4(2)	0.4(2)	-	0.2(2)	0.1(2)	-
Citrate	0.4(2)	0.3(2)	-	0.2(2)	0.1(2)	-
Iso-citrate	0.3(2)	0.3(2)	-	0.2(2)	0.1(2)	-
Proline	0.4(3)	0.7(2)	-	0.2(2)	0.1(2)	-

Figures in parentheses denote the number of analyses, of which the mean value is given.

decreased to 0.39 - 0.46 measured under identical incubation conditions. This coincided with the gradual deterioration of the pleomorphism of the EATRO 173 strain, described in Chapter 2.

The pattern of utilisation of the substrates listed in Table 2 is very similar to that of the culture form of T.rhodesiense (Ryley, 1962), with the exception that the pleomorphic bloodstream forms cannot utilise exogenous succinate. The rate of oxygen uptake by the culture forms utilising succinate is stimulated 6 - 7 fold by incubation in an acidic medium; however, no significant increase in the utilisation of this substrate by the bloodstream organisms occurred at pH 5.6, and the glucose supported respiration was markedly decreased, as follows. The data of Ryley (1962) on the cultured organisms are included for comparison:

			Oxygen uptake as $\mu l\ O_2/min/mg N$	
Substrate:			Glucose	Succinate
T.rhodesiense EATRO 173 bloodstream forms	pH	7.4	17.7 (12)	0.46 (2)
	pH	5.6	6.2 (2)	0.49 (2)
Culture forms (Ryley 1962)	pH	7.5	8.0	2.2
	pH	5.6	* 100%	* 175%

Figures in brackets indicate number of experiments.

*Absolute data not reported.

Although the incubation conditions employed by Ryley differ slightly from those employed in this work, (the figures for the culture forms were obtained at 30°C with a gas phase of CO_2 + air (5 : 95), as opposed to the standard conditions of 37°C and a gas phase of air used for the bloodstream organisms) it may be seen that the capacity for succinate utilisation is not present in the SS forms.

TABLE 3

Oxygen utilisation by standard water-lysed preparations in the presence of various substrates

Standard water-lysed material (approx. 1.0 mg N) was suspended in MFM with the substrates as indicated at 20 µmoles/flask. Manometric details as described in Chapter 3 and Table 2. Where a substrate is marked with an asterisk*, NADP (5 µmoles) was included in the incubation media. All values are corrected for control values obtained in the absence of exogenous substrate. Pleomorphic composition of EATRO 173 = 78 - 83% SS.

	T.rhodesiense EATRO 173			T.brucei TREU 277		
	O_2 uptake	CO_2 evolution	R.Q.	O_2 uptake	CO_2 evolution	R.Q.
	(µl / min / mg N)			(µl / min / mg N)		
Glucose	4.49(7)	2.29(7)	0.51	4.91(3)	0.4(3)	0.1
∝-Oxoglutarate	2.12(11)	4.18(10)	1.97	0.3 (6)	0.2(4)	-
Pyruvate	1.67(9)	3.09(7)	1.85	0.3 (6)	0.2(3)	-
L- ∝-GP	5.12(3)	1.18(2)	0.23	4.94(2)	0.4(2)	0.1
Succinate	0.5 (4)	0.1 (4)	-	0.3 (2)	0.2(2)	-
Citrate*	0.3 (2)	0.5 (2)	-	0.3 (2)	0.2(2)	-
Iso-citrate*	0.6 (2)	0.5 (2)	-	0.3 (2)	0.2(2)	-
Fumarate*	0.2 (2)	0.1 (2)	-	0.3 (2)	0.2(2)	-
Aspartate	0.2 (2)	0.1 (2)	-	0.3 (2)	0.2(2)	-
Proline	0.6 (2)	0.3 (2)	-	0.3 (2)	0.2(2)	-
Malate*	0.2 (2)	0.1 (2)	-	0.3 (2)	0.2(2)	-

Figures in parentheses denote the number of analyses, of which the mean value is given.

Utilisation of metabolites by lysed cell preparations.

While experiments using whole organisms give some indication of the cellular properties in vivo, the selective permeability of the organisms to substrates may obscure the ability of the cells to utilise the endogenously produced compounds. The standard water-lysed preparation was therefore used to investigate the capacity of the organisms to utilise various energy sources (Table 3).

The rate of oxygen uptake by the water-lysed preparation utilising L-α-GP is about half the rates obtained by Grant et al., (1961). However, these authors used a partially purified system, consisting of the resuspended pellet from a repeatedly water-washed lysate, and much nitrogenous material was presumably lost in the discarded supernatants.

A comparison of the data in Tables 2 and 3 also shows that the rate of oxygen uptake in the presence of glucose is reduced by 75% by the process of cell lysis. As a lowering of the efficiency of the system is unavoidable on disrupting the co-ordinated intracellular structure, no further attempt was made to improve the general assay system.

The negligible utilisation of pyruvate by the intact cells of EATRO 173, appears to be due to a permeability barrier preventing access of this metabolite to the pyruvate oxidase system. The development of this system is confined to the pleomorphic strain in the same manner as the α-oxoglutarate oxidase system and the R.Q. values for pyruvate and α-OG suggest that their catabolism is confined to one step oxidative decarboxylation reactions.

The low utilisations of the amino-acids proline and aspartate, and of the tricarboxylic acid cycle intermediates succinate, citrate, isocitrate, fumarate and malate by the pleomorphic strain indicate minimal

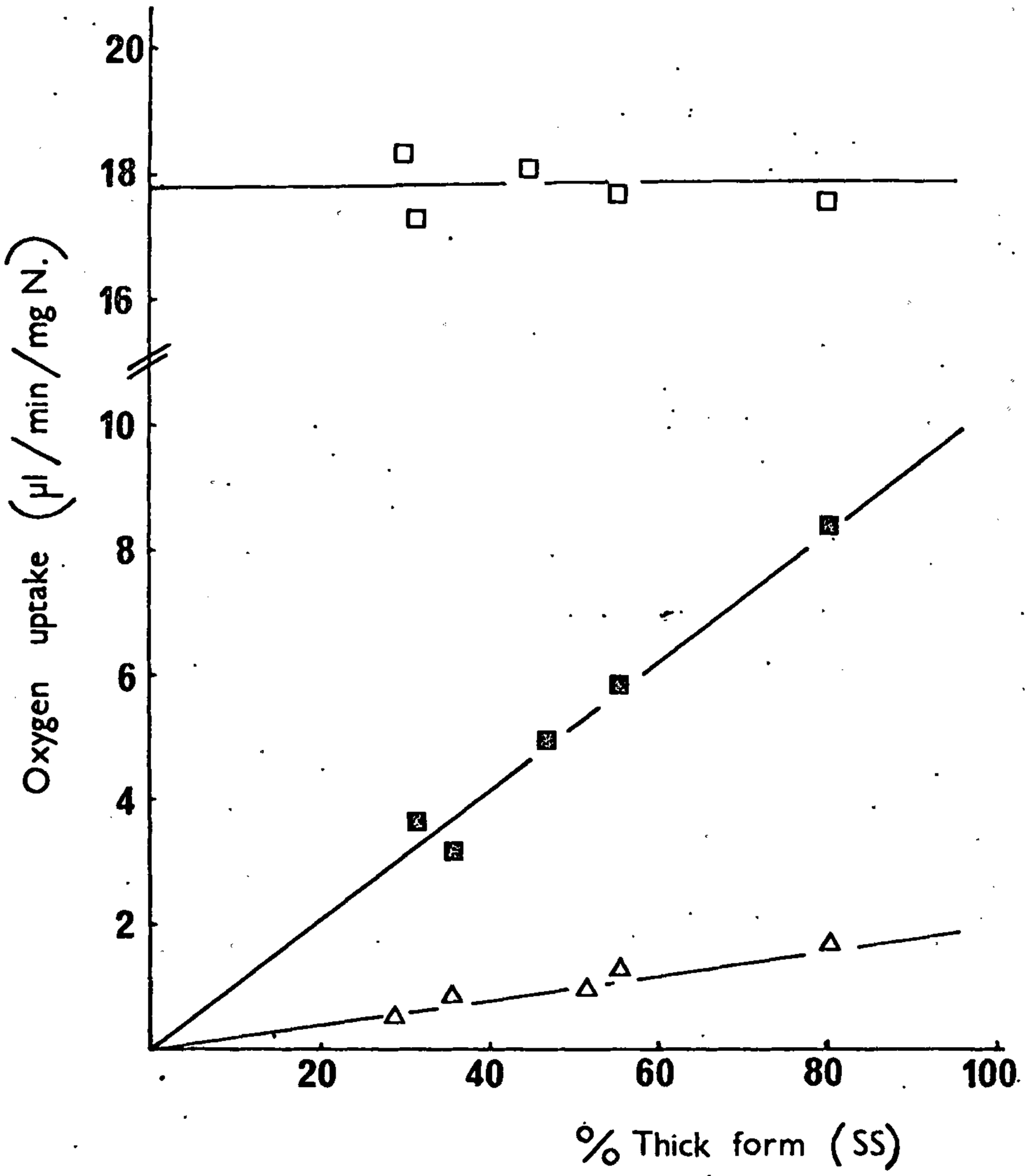


Fig. 6. The rate of oxygen utilisation by T. rhodesiense EATRO 173 as a function of the pleomorphic composition. Whole cells (approx. 0.2mg N) were suspended in Krebs saline with 20 µmoles glucose (□) or ∝-oxoglutarate (■) and incubated under the standard manometric conditions as described in Chapter 3. Standard water-lysed preparations (approx. 1.0mg N) were suspended in MFM with 20 µmoles pyruvate (△) and incubated under the same conditions. The utilisation of oxygen was measured from 10 to 40 minutes after the start of the experiments. All data are corrected for the oxygen uptake obtained in the absence of exogenous substrate, and each point represents the mean of three determinations.

cycle activity under the conditions employed. No R.Q. figures are given for these substrates as the total gas exchanges are of the order of 3 - 15 µl over the experimental period. At this level of estimation, manometric measurement is accurate to not more than ± 1 µl, or 6 - 30%. A small error of reading thus introduces a discrepancy which is large enough to make a calculated R.Q. meaningless.

Dependence of the substrate utilisation upon the proportions of cell types.

The simple motility test indicated the preferential utilisation of α-OG by the SS forms of EATRO 173, and this was borne out by manometric observation, as shown in Figure 6. Organisms were harvested at different stages of the parasitaemia, corresponding to different SS/LS ratios and the rate of oxygen uptake in the presence of α-OG or glucose was measured. The utilisation of α-OG may be concluded from these data to be a specific property of the SS forms. When similar experiments were carried out with standard water-lysed preparations, it was found that the presence of the pyruvate oxidase system was similarly confined to the SS organisms (Figure 6).

The mechanism of pyruvate and α-oxoglutarate utilisation by cell lysates.

In an attempt to demonstrate whether the utilisations of pyruvate and α-OG are by classical oxidase systems, further supplementation of the MFM was carried out, with the results as shown in Table 4. Standard water lysed preparations (approx. 1.0 mg N) were suspended in the media indicated and the gas exchanges were measured over a period of 30 minutes, after addition of 20 µmoles pyruvate. None of the cofactors was found to significantly stimulate utilisation, but this does not preclude the

TABLE 4

Effect of supplementation on gas exchange by standard water-lysed preparations utilising pyruvate

Experimental conditions as described in Table 3. Results are corrected for the gas exchange in the absence of exogenous pyruvate, in the presence of the relevant cofactors. Values are calculated on a basis of MFM alone = 100 and are the mean values with the standard deviations.

Organism: T.rhodesiense EATRO 173. Pleomorphic composition 79-81% SS.

	Cofactor additions	O_2 uptake	CO_2 evolution
1)	MFM	100	100
2)	MFM, NADP (5 µmoles), Coenzyme A, TPP, Lipoic acid (reduced), (1 µmole each).	105 ± 3 (5)	101 ± 3 (5)
3)	As 2) + Fe SO_4 (3 µmoles) + GDP (5 µmoles)	106 ± 3 (3)	98 ± 4 (3)
4)	As 3) + aspartate (5 µmoles)	93 ± 4 (3)	96 ± 4 (3)
5)	As 3) + oxaloacetate (2 µmoles)	100 ± 3 (3)	106 ± 4 (3)
6)	As 3) + malate (2 µmoles)	104 ± 3 (4)	107 ± 4 (4)

Figures in parentheses indicate the number of estimations.

operation of oxidase systems similar in nature to those found in mammalian systems. Even after the final 48-fold dilution of the cell lysate, sufficient coenzyme A may be present to prevent stimulation by further addition of the cofactor. Hydrolysis of acetyl coenzyme A, or the absence of citrate synthase (E.C. 4.1.3.7.), similarly would abolish any stimulation of the gas exchange rates.

Removal of endogenous cofactors by dialysis of water-lysed preparations for 2 - 4 hours against two changes of 0.01 M-tris buffer pH 7.1 led to extremely low yields of activity of both the pyruvate and α-OG oxidase systems. After complete refortification with MFM plus NADP, coenzyme A, thiamine pyrophosphate and lipoate, activity of these systems ranged from 0-14% of the rates obtained with untreated lysates. As the only oxygen utilising system known to be present in these organisms is the L-α-GP oxidase complex, 2 μmoles of FDP (as a source of DHAP) was added to the dialysates, but did not stimulate the rates of oxygen uptake.

The use of G.25 Sephadex was more successful in the removal of endogenous cofactors, although the glutinous nature of the lysates limited the yields from gel filtration (c.f. purification of pyruvate kinase, Chapter 5). Standard water-lysed preparations were applied to a column of G.25 Sephadex (10 cm x 1.5 cm diameter) pre-equilibrated in physiological saline, and fractions (2 ml) were collected on elution with saline. It was necessary to stir the top of the column of gel with a glass rod to avoid complete agglutination of the material; even with this treatment the yield of total protein through the column was less than 25% of the application. The rates of oxygen utilisation obtained with totally augmented preparations of this nature, using pyruvate, were extremely variable, and ranged from 18 - 80 nmol

TABLE 5

The effect of supplementation on oxygen uptake by Sephadex - treated standard water-lysed preparations

Cell lysates were desalted as described in the text. Aliquots (2.0 ml, approx. 1.0 mg N) were incubated in the oxygen electrode system with 1.0 ml of MFM containing 20 µmole substrate. Cofactors were added sequentially as indicated (in a volume of 10 µl). All rates are corrected for control values obtained in the absence of substrate.

Organism: T.rhodesiense EATRO 173. Pleomorphic composition 80-86% SS.

	Addition	Amount of fortification (µmoles)	Rate (nmol O_2/10"/mgN)
1)	Pyruvate	20	4.4
	NAD^+	5	4.6
	ADP	5	6.2
	CoA	1	55.8
	Lipoate (reduced)	1	56.2
	TPP	1	58.2
	Malate	2	61.4
	Oxaloacetate	2	62.1
2)	Pyruvate	20	8.9
	CoA	1	20.2
	Lipoate (reduced)	1	21.3
	TPP	1	21.6
	NAD^+	5	41.5
	ADP	5	61.8
	Malate	2	62.6
	NADP	5	64.2
3)	∝-oxoglutarate	20	14.3
	CoA	1	38.2
	Lipoate (reduced)	1	37.4
	TPP	1	37.4
	NAD^+	5	75.1
	ADP	5	116.4
	GDP	5	108.2

O_2/10 min/mg N i.e. never greater than approximately 10% of the rates obtainable with whole lysates. The Clark oxygen electrode, having a higher sensitivity, was therefore used to measure the rates of oxygen utilisation. The effects of further fortification, estimated by sequential addition of cofactors, are shown in Table 5. It must be stressed that under the conditions of these assays, the utilisation of oxygen is occurring under conditions where the L-α-GP oxidase system is expected to be inactive i.e. in the absence of DHAP as a hydrogen carrier. The positive utilisations obtained could imply incomplete removal of DHAP, or the presence of an alternative oxygen-utilising NADH oxidase. The reproducible stimulation of oxygen uptake by ADP supports the latter possibility, as the L-α-GP oxidase system is reported as being ADP-independent and non-phosphorylating (Bide, 1963). On the other hand, the partial stimulation by coenzyme A in the absence of exogenous NAD^+ and ADP may indicate incomplete removal of these two cofactors, thus strengthening the possibility that a catalytic trace of DHAP may also still be present. The complete removal of coenzyme A, however, appears to be achieved by the gel filtration.

NAD^+, ADP and coenzyme A are the only cofactors which stimulate oxidation of pyruvate (Table 5). The failure of TPP and lipoate to increase the rates of oxygen uptake, corresponds with the known properties of mammalian α-keto acid oxidases in which these cofactors are prosthetically bound, thus making Sephadex treatment ineffectual for their removal. The lack of stimulation by malate and NADP is probably due to the extremely low levels of citrate synthase activity in the extracts, as malate dehydrogenase is very active in these preparations.

2) CYTOCHROME PIGMENTS IN T.RHODESIENSE.

The interpretation of the above results, while subject to a number of reservations, suggested the existence of a terminal oxidase system alternative to the L-α-GP oxidase, in the pleomorphic organisms. Monomorphic trypanosomes have repeatedly been examined for the presence of cytochromes, and for cyanide and carbon monoxide sensitivity, with negative results (Ryley, 1956; von Brand, 1951; Fulton and Spooner, 1959). With the development of the α-keto acid oxidase systems in the pleomorph, the possibility of the presence of the cytochrome system in the SS forms had to be considered.

Whole cell preparations and lysates of the pleomorph were subjected to spectrophotometric analysis with NADH, glucose and dithionite as reducing agents. Reduced versus oxidised spectra of these preparations did not show the presence of cytochrome pigments.

The more sensitive chemical estimation of haem pigments by pyridine haemochromogen formation was attempted on lysed material and on lyophilised preparations, again with negative results. Some early samples of lyophilised T.rhodesiense showed the presence of trace amounts of a pyridine haemochromogen corresponding to ferroprotoporphyrin IX (V_{max} 423 nm; 558 nm) but these traces are eliminated by rapid removal of the erythrocytes from infected blood samples, ensuring minimal red cell lysis during the preparation of the trypanosomes. Presumably these traces are therefore due to contaminating haemoglobin.

In agreement with Ryley (1962), the conclusion is that if cytochrome pigments are present in the blood stream organisms, they are to be found only in exceptionally small concentrations. Assuming a molar extinction coefficient of 2×10^4 for the Soret bands of the pigments (Williams 1964), the estimation method used should have detected the

presence of absorbing pigments at amounts $\leqslant$ 0.2 nmole per 10 mg protein. This compares with the figure of 1.14 nmole protoheme per 10 mg protein found to be present in C.fasciculata (Hill & White 1968).

The preparation of trypanosomal material free from haem complexes does, however, demonstrate the purity of the organisms from blood elements.

Succinate dehydrogenase and citrate synthase in T.rhodesiense.

Several enzymes of the tricarboxylic acid cycle have been demonstrated in monomorphic T.rhodesiense, as described in Chapter 1. This work adds the two oxidative decarboxylase systems of the cycle in pleomorphic infections. Hence, the only cycle enzymes which have not been demonstrated are citrate synthase and the succinoxidase system. Repeated attempts to demonstrate the synthesis of citrate by lysed cell preparations in which the oxidation of pyruvate was supplemented by aspartate, oxaloacetate or malate gave negative results, when citrate was estimated by the method of Siebert (1965). When the direct assay for activity of this enzyme was employed, however, low levels of activity were estimable.

Succinoxidase activity was also demonstrated, by use of the polarographic estimation of oxygen utilisation with succinate as the substrate. The rates obtained for oxygen utilisation in the presence of 20 µmoles succinate were approx. 5 nmol O_2/min/mg N, i.e. about 5% of the rate of α.oxoglutarate oxidation. Apart from the demonstration that this enzyme is competitively inhibited by malonic acid, no further investigation of the system was carried out. Sonication or freeze-thawing as means of cell lysis resulted in even lower levels of succinoxidase activity.

TABLE 6

The effect of metabolic inhibitors on the oxygen uptake of T.rhodesiense EATRO 173

Whole cells (approx. 0.2 mg N) or standard water-lysed preparations (approx. 1.0 mg N) were incubated in saline and MFM respectively, in the presence of varying concentrations of the inhibitors. Oxygen uptake was measured over a 30 minute period and values are expressed as mean I_{50} values $\pm$ standard deviations. Each I_{50} value is from a series of at least five inhibitor concentrations. Other experimental details are given in Chapter 3.

	I_{50} (M)						
Subtrate:	Glucose		∝-oxoglutarate		Pyruvate	∝-GP	Concentration at which "no inhibition" is reported (M)
Inhibitor	whole cells	Lysate	whole cells	Lysate	Lysate	Lysate	
Rotenone	$1.3\pm0.3\times10^{-5}$(6)	$1.0\pm0.2\times10^{-5}$(4)	$5\pm2\times10^{-6}$(4)	$4.0\pm0.9\times10^{-6}$(5)	$5.1\pm1.1\times10^{-6}$(5)	N.I.	10^{-5}
Amytal	$1.2\pm0.4\times10^{-3}$(3)	$3.0\pm0.8\times10^{-3}$(3)	$9.0\pm1.5\times10^{-4}$(6)	$1.9\pm0.8\times10^{-3}$(4)	$1.1\pm0.2\times10^{-3}$(3)	N.I.	3×10^{-3}
Cyanide	N.I.	N.I.	N.I.	$5.0\pm0.6\times10^{-4}$(7)	N.I.	N.I.	5×10^{-4}
Azide	N.I.	N.I.	*	*	N.I.	N.I.	10^{-3}
Antimycin A	N.I.	N.I.	N.I.	N.I.	N.I.	N.I.	6 µg/ml
Malonate	N.I.	N.I	N.I.	N.I.	N.I.	N.I.	10^{-2}

N.I. denotes not inhibited.

* denotes approx. 30% inhibition at 10^{-3} M azide.

Figures in parentheses denote number of results.

3) THE EFFECT OF METABOLIC INHIBITORS ON PLEOMORPHIC T.RHODESIENSE.

Whole cell or standard water-lysed preparations were incubated in the standard Warburg system in the presence of varying concentrations of the inhibitors shown in Table 6. The fragility of the whole organisms precluded preincubation of the cells with inhibitors in the absence of substrate, and for comparative purposes substrate was also included in the lysate systems during preincubation. In the presence of higher concentrations of some inhibitors, the inhibition increased with time, and in these cases, the initial rates of oxygen uptake were estimated.

The general lack of inhibition by cyanide indicates the absence of a functional cyanide-binding cytochrome oxidase in these organisms, and the inability of azide and antimycin A to inhibit oxygen uptake, similarly indicates the absence of cytochrome oxidase and the cytochrome (b→c) electron transfer step respectively. Azide was also tested at pH 6.5, since it has been stated that this inhibitor is inactive against the electron transport chain at pH > 7 (Keilin 1936). At pH 6.5, the utilisation of glucose was depressed, but azide was still ineffective.

The inhibition by cyanide in the case of cell lysates utilising α-OG is somewhat anomalous. Cyanide complexes with α-keto acids, but in the concentrations used (cyanide: α-OG approx. 1: 14) this should not have decreased the available substrate significantly, and this removal of substrate would not account for the differential susceptibilities of whole cells and lysates to the inhibitor. If this inhibition is via a specific action of cyanide on the electron transport system between α-OG and oxygen, the implications are that two routes for oxygen utilisation are present, that these routes are utilised preferentially by different substrates, and that <u>in vivo</u> a permeability

TABLE 7

Effect of rotenone on NADH utilisation by Sephadex G-25 treated cell lysates

Experimental material prepared and used as in Table 5. Polarographic rates are corrected for oxygen utilisation in the absence of exogenous substrate, and are expressed as nmol O_2/10min/mg N. Rates obtained in the presence of FDP are corrected for the oxygen utilisation in the presence of this metabolite alone. Rotenone was added to a final concentration of 10^{-5} M, in 10 µl ethanol.

Substrate	Rate of oxygen utilisation (nmol/10min/mg N)	% Inhibition
NADH (5 µmoles)	22.6	-
NADH (5 µmoles) + ethanol	22.4	1
NADH (5 µmoles) + ethanol + rotenone	6.7	70
NADH (5 µmoles) + FDP (2 µmoles)	241	-
NADH (5 µmoles) + FDP (2 µmoles) ± ethanol	214	11
NADH (5 µmoles) + FDP (2 µmoles) + rotenone ± ethanol	63.8	74

barrier to cyanide exists. Alternatively, the in vivo reoxidation of NADH from α-OG metabolism is entirely via the cyanide insensitive pathway.

Amytal and rotenone were thought to be specific inhibitors of the flavoprotein / non-haem ferroprotein electron transfer step in the cytochrome chain, oxidising NADH. Recent reports, however, have extended the effects of rotenone to include focal points other than site I of the electron transport system. Butow [ ] (1966), showed beef liver glutamate dehydrogenase to be rotenone sensitive, and Balcavage [& Mattoon ?] (1967) demonstrated rotenone inhibition of yeast mitochondrial alcohol dehydrogenase. In the absence of demonstrable cytochrome pigments in pleomorphic T.rhodesiense, the rotenone/amytal sensitive site presumably resembles these enzymic systems in being extra-cytochromal. The focal point of the inhibition must either be associated with the L-α-GP oxidase system, or coupled to an alternative autoxidisable system.

NADH oxidation by desalted cell lysates.

Standard water-lysed preparations of T.rhodesiense EATRO 173 were treated as described previously in an attempt to remove endogenous DHAP and thus render the L-α-GP oxidase system inactive. Such preparations still utilised NADH at the expense of molecular oxygen, although readdition of FDP increased the rate of oxygen uptake approx. 10-fold (Table 7). This stimulation was remarkably constant in the preparations used, (ranging only between 9.2-fold and 11.1-fold in seven Sephadex-treated samples), which may indicate the presence of two separate systems. If this is the case, however, both systems are rotenone sensitive. As L-α-GP oxidase is not sensitive to rotenone (Table 6), the NAD^+-linked L-α-GP dehydrogenase appeared to be a possible site for the action of this inhibitor. This was supported by the similarity of inhibitor

TABLE 8

Preliminary carbon balance studies on the metabolism of glucose by whole cells.

Whole organisms (0.3 mg N) were suspended in saline with 25 µmoles glucose per flask, and incubated under the standard manometric conditions. Aliquots of the deproteinised flask contents were analysed for glucose and pyruvate. Results are expressed on the basis of the net experimental metabolism after consideration of the zero time samples (see Chapter 3). The experimental periods varied from 35 to 60 minutes as indicated.

		% glucose carbon found			
Strain	Pleomorphic composition	CO_2	Pyruvate	Total	Experimental duration(mins)
T.rhodesiense "L" strain	Monomorph	0.1	89.0	89.1	45
T.brucei TREU 277	72% thick	5.0	56.9	61.9	35
T.brucei TREU 277 (passaged)	Monomorph	2.3	74.0	76.3	40
T.rhodesiense EATRO 165a	80% thick	6.5	44.1	50.6	60
T.rhodesiense EATRO 165a	32% thick	1.5	52.1	53.6	60

concentrations required for 50% inhibition of oxygen utilisation with glucose, pyruvate and α-OG as substrates, indicating a common site of action independent of the NADH source. Unfortunately, a final check on this hypothesis, in which the NAD^+-dependent dehydrogenase was assayed spectrophotometrically, showed it to be untenable. Lyophilised trypanosomes desalted on G-25 Sephadex, were resuspended in 0.01 M-tris buffer pH 7.3 at 20 mg/ml, and the rate of NADH reoxidation by the preparation was measured at 340 nm. In this system, oxidation of NADH alone was extremely slow, but was rotenone sensitive, whereas on addition of FDP the oxidation rate was stimulated 20-fold and was rotenone insensitive.

At this point it was found that concentrations of rotenone and amytal in the same range as those used with the pleomorphic organisms, were as effective in inhibiting oxygen uptake by the monomorphic strain utilising glucose. The action of these inhibitors is not therefore indicative of a developmental change in the slender to stumpy transformation, and no further investigation of the system was carried out.

4) CARBON BALANCE STUDIES ON PLEOMORPHIC T.RHODESIENSE.

The lack of detectable cytochrome pigments in the pleomorphic strains of T.rhodesiense, indicated that mitochondrial differentiation in these organisms is not complete. However, the presence of pyruvate and α-OG oxidase systems, and the production of significant amounts of carbon dioxide from the metabolism of glucose, suggested that the tricarboxylic acid cycle may be (partially) functional at this stage of the cyclical development.

Preliminary estimates of the end-products of glucose metabolism are shown in Table 8. Whereas the recovery of glucose carbon as pyruvate is approaching quantitative levels in the "L" strain, between

TABLE 10

Carbon balance studies on T.rhodesiense EATRO 173: effect of the incubation period

Experimental conditions as in Tables 8 and 9. Substrate: 25 µmoles glucose. Material: whole cells (approx. 0.3 mg N).

	%SS forms present	Experimental duration (minutes)	Pyruvate	CO_2	Glycerol	Succinate	Acetate	Total
1.	79	45	62	9	8	7	9	95
2.	81	45	65	8	7	4	8	92
3.	81	50	60	9	8	6	9	92
4.	78	50	56	9	10	8	9	92
5.	83	60	56	10	8	7	10	91

TABLE 9

Carbon balance studies on T.rhodesiense EATRO 173: range of products

Experimental details regarding whole cells, as in Table 8. Standard water-lysed material (10 mg N) was suspended in MFM with 25 µmoles pyruvate. Substrate utilisation and metabolite formation were estimated on deproteinised samples as in Chapter 3. Yields are expressed as percentages of the glucose carbon utilised. Figures in parentheses indicate the number of determinations. Pleomorphic composition: 77% - 80% SS.

	% carbon found		
	Whole organisms		Lysate
Substrate:	Glucose	α-oxoglutarate	Pyruvate
Product			
Pyruvate	56-65 (8)	2 (2)	-
CO_2	7-12 (8)	20-22 (2)	30 (1)
Succinate	4-10 (5)	73-76 (2)	1 (1)
Glycerol	5-10 (7)	N.E.	1 (1)
Acetate	8-10 (5)	1 (2)	62 (1)
Citrate	1 (2)	1 (2)	1 (1)
Hexose phosphate	1 (2)	N.E.	N.E.
PEP	2 (3)	N.E.	Zero (1)

N.E. denotes not estimated.

26% and 56% is unaccounted for in the other strains used. The carbon dioxide yield appears to be related to the percentage of SS forms and continual passage leads to decreased carbon dioxide production and an increased yield of pyruvate.

The pyruvate yield from the EATRO 165a strain was consistently lower than that obtained from the major pleomorphic strain used in this work, EATRO 173, although the carbon dioxide yield was also slightly lower (cf. Table 9). No significance other than natural biological variation can be attached to these differences.

In an attempt to elucidate the pathways involved in the metabolism of glucose, further balance studies were carried out, involving a more comprehensive series of possible end-products (Table 9).

Catabolism of glucose, whilst partially terminating at pyruvate, does produce significant although relatively minor quantities of acetate, glycerol and succinate. Although the total recovery of glucose carbon was relatively constant at 91 - 95%, in the five analyses where all metabolites were estimated on a single incubation (Table 10), there was considerable scatter in the percentage accumulation of the various products. These variations did not correspond to the minimal differences in pleomorphic composition of the preparations used, and the only parameter which varied between experiments was the duration of the experimental period.

Effect of incubation time on metabolite production from glucose.

At the time these experiments were carried out, T.rhodesiense EATRO 173 had been stored in stabilate form at -70°C for between 7 and 12 months. Some deterioration of the pleomorphism appeared to have occurred as previously described, reflected in relatively higher yields of pyruvate than those obtained with the newly acquired material. The CO_2 production and R.Q. values obtained were also lower after storage.

The absolute values for metabolite formation at various incubation

TABLE 11

Effect of incubation time on metabolite formation from glucose

Whole cells (approx. 0.3 mg N) of T.rhodesiense EATRO 173 were incubated under standard manometric conditions in the presence of 20 µmoles glucose as substrate. At the indicated times after removal of the zero-time samples, pairs of reaction flasks were removed for metabolite analysis. Pleomorphic composition: 78 - 82% SS.

Experimental duration	Glucose utilised (µmoles)	O_2 utilised (µmoles)	O_2/glucose	Metabolite produced (µmoles)					R.Q.	Total recovery %
				Pyruvate	CO_2	Acetate	Succinate	Glycerol		
10	2.40	2.88	1.20	3.27	0.72	0.62	0.14	0.32	0.25	92
10	1.92	2.31	1.20	2.82	0.46	0.60	0.06	0.19	0.20	95
20	3.16	3.73	1.18	5.56	1.14	0.85	0.19	0.32	0.30	96
20	3.95	4.59	1.16	5.06	1.18	0.95	0.24	0.59	0.26	88
30	5.05	5.76	1.14	6.07	2.42	1.31	0.45	0.71	0.42	90
30	4.10	4.72	1.15	5.31	1.48	1.31	0.31	0.60	0.31	93
45	7.42	8.02	1.08	9.19	4.04	2.01	0.78	1.21	0.50	95
45	8.16	8.56	1.05	10.61	3.96	1.98	0.50	1.13	0.46	92
50	8.04	8.35	1.04	9.64	4.34	2.22	0.72	1.28	0.52	92
50	8.35	8.85	1.06	10.01	3.51	2.01	0.75	1.80	0.40	92
50	6.71	7.05	1.05	8.00	2.78	1.80	0.71	1.20	0.39	96
60	8.31	8.38	1.01	9.31	4.64	2.18	0.96	1.61	0.55	92
60	8.82	8.86	1.00	9.95	5.35	2.59	0.90	1.32	0.60	91
60	10.00	10.10	1.01	10.82	4.62	2.41	1.22	2.18	0.45	89
70	9.42	9.92	1.05	10.61	4.93	2.83	1.27	1.52	0.50	92

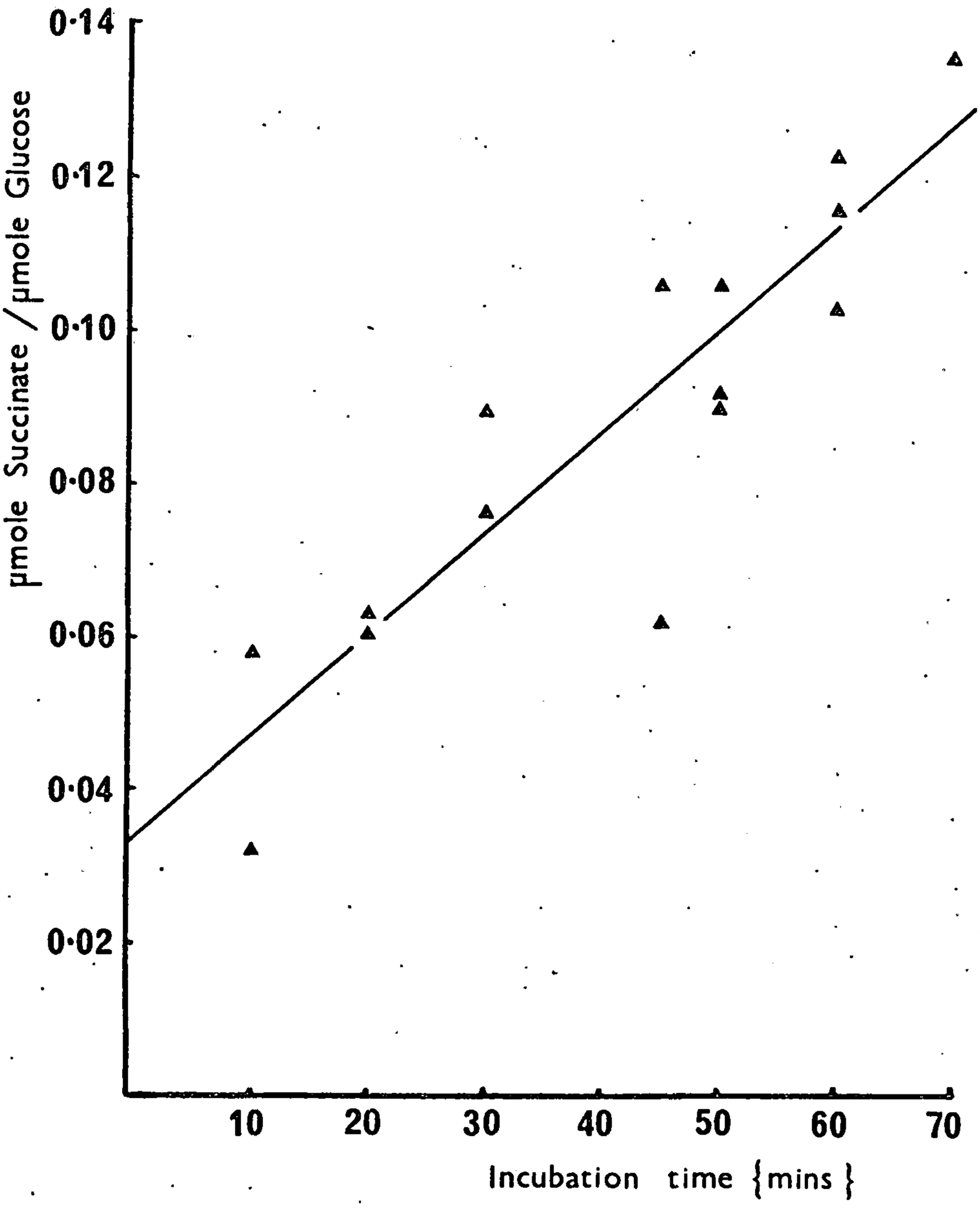


Fig. 7. The effect of incubation time on the glucose metabolism of whole cells.

d). The production of succinate.

The data of Table 11 expressed as molar ratios with respect to the utilisation of glucose.

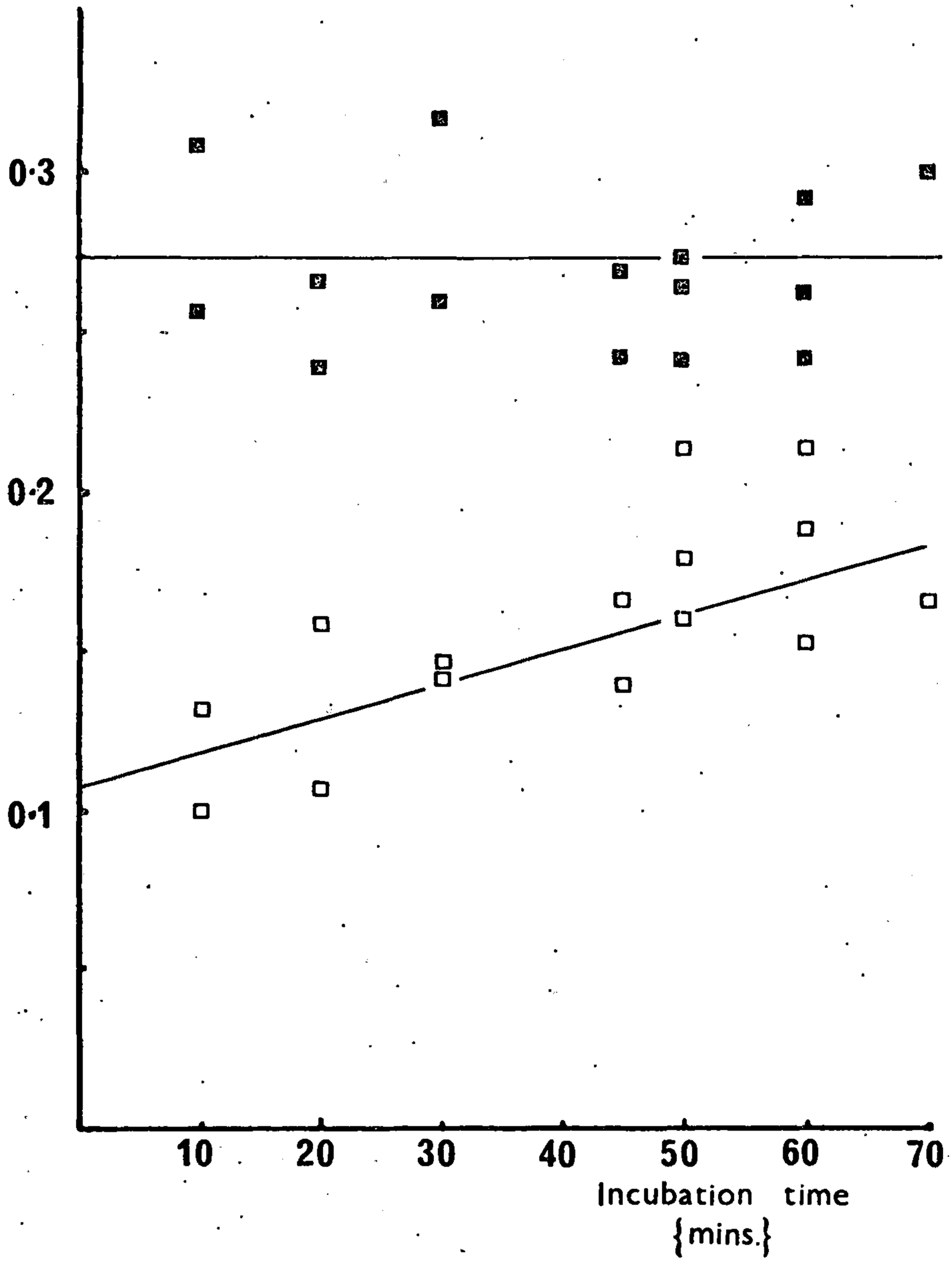


Fig. 7. The effect of incubation time on the glucose metabolism of whole cells.

c). The production of acetate and glycerol.

The data of Table 11 expressed as molar ratios with respect to the utilisation of glucose.

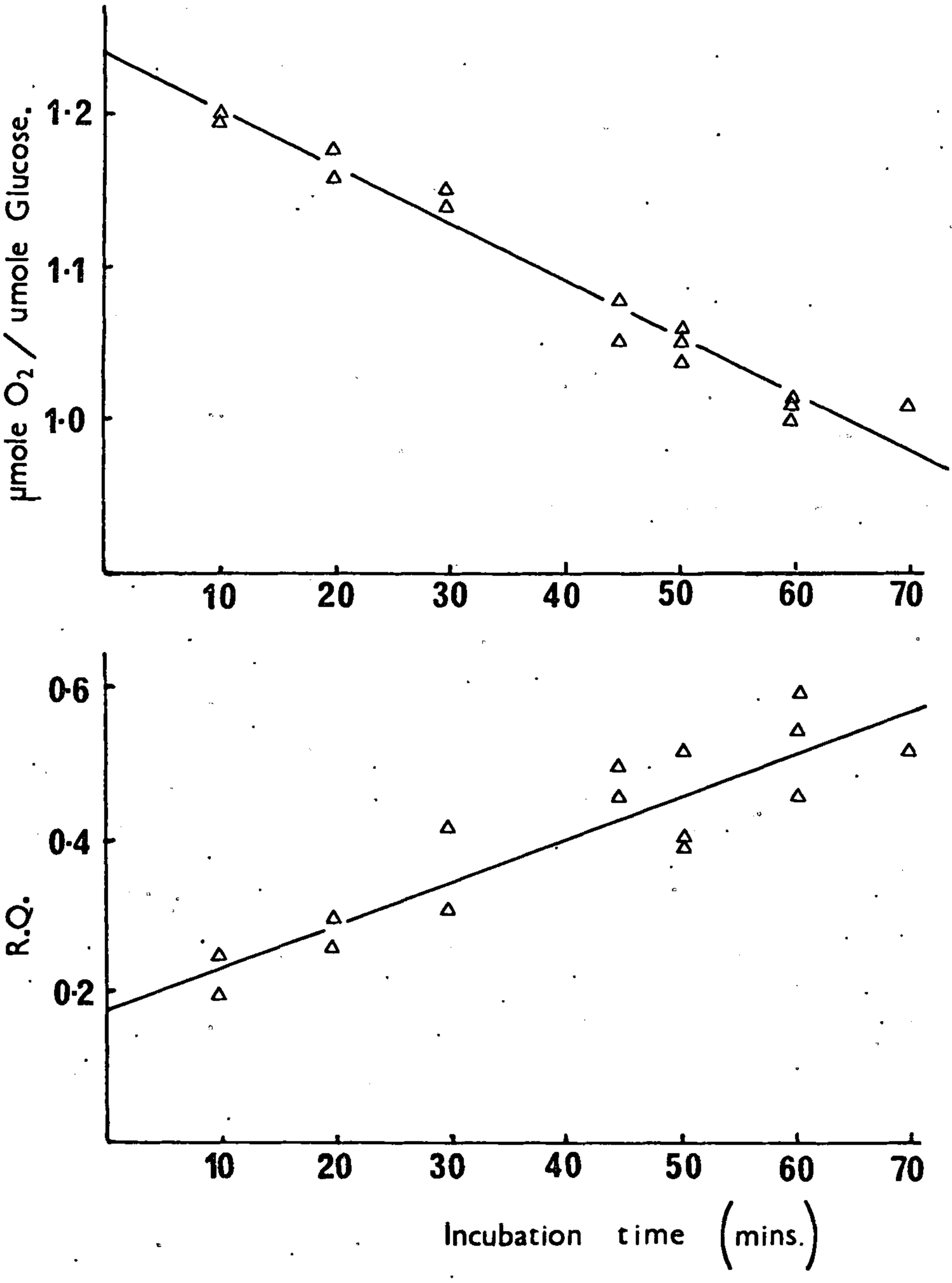


Fig. 7. The effect of incubation time on the glucose metabolism of whole cells.

a). The R.Q. value and the utilisation of oxygen. The utilisation data of Table 11 are expressed as molar ratios with respect to the glucose utilisation.

times, are given in Table 11, and are plotted in terms of /umole metabolite/ /umole glucose utilised, in Figure 7 (a-d). Over a period of 70 minutes, pyruvate production and oxygen utilisation per glucose utilised steadily decrease, whereas % yields of glycerol, succinate and CO_2 increase. The ratio of acetate produced per glucose utilised remains constant.

The decrease in oxygen consumption may be qualitatively accounted for by the parallel increase in glycerol production. Dephosphorylation of L-α-GP to produce glycerol will enable the reoxidation of NADH by DHAP to occur, without a concomitant utilisation of oxygen. The increase in production of carbon dioxide and succinate at the expense of a decreased yield of pyruvate, may reflect an effect of the increasing pool size of pyruvate during incubation and its further catabolism by decarboxylation. After one hour, the in vitro concentration of pyruvate is approximately 3 mM, whereas the normal uninfected range of blood pyruvate concentrations in vivo is 0.2 - 0.4 mM (von Brand & Coleman, 1957). The same authors report that even in heavily infected experimental animals, no significant change in the blood pyruvate level occurs until the immense terminal parasitaemia is reached.

Effect of carbon dioxide on metabolite production from glucose.

Throughout the work reported above, the manometric technique used involved the inclusion of incubation flasks in pairs (i.e. with and without KOH in the centre well) in order to measure oxygen uptake and carbon dioxide production simultaneously. In some cases the contents of such pairs of flasks were mixed prior to analysis, to increase the volume available for duplication of estimation; the question therefore arose as to whether the absence of KOH (i.e. the presence of CO_2) during

TABLE 12

Comparison of manometric systems for investigation of glucose metabolism

Experimental conditions as in Table 8. Metabolite yields are given both as μmoles and as % of glucose utilised. The data from two experiments are presented. Organism - whole cells T.rhodesiense EATRO 173. Pleomorphic composition 82 and 80% SS. Incubation periods 45 and 40 minutes respectively.

	Experiment A						Experiment B					
	Double side-arm		Single side-arm				Double side-arm		Single side-arm			
			+KOH		-KOH				+KOH		-KOH	
	(μmoles)	(%)	(μmoles)	(%)	(μmoles)	(%)	(μmol)	(%)	(μmol)	(%)	(μmol)	(%)
Utilisation												
Glucose	7.79		7.65		7.71		7.14		7.01		7.06	
Oxygen	7.72		7.80		-		7.19		6.90		-	
Production												
Pyruvate	9.65	62	9.32	61	9.42	61	8.74	61	8.37	60	8.20	58
Glycerol	1.52	10	1.50	10	1.49	10	1.41	10	1.26	9	1.42	10
CO_2	2.68	6	-	-	2.60	6	2.42	6	-	-	2.48	6
Succinate	0.51	4	0.50	4	0.51	4	0.48	5	0.46	4	0.48	5
Acetate	1.65	7	1.64	7	1.66	7	1.48	7	1.51	7	1.46	7
Total carbon yield		89%				88%		89%				86%

incubation also affected the course of metabolism. Any differences between such a pair of flasks would produce anomalous values for the gas exchange occuring, as the evolution of carbon dioxide in the flask without KOH is measured on the basis of the oxygen utilisation in the other member of the pair. To clarify this possibility, flasks were obtained with two side-arms, one of which is connected to a trough-shaped centre vessel. It is possible in these systems to measure oxygen utilisation and carbon dioxide evolution on the same experimental flask, by the production of a carbon dioxide absorbing solution in situ at the end of the incubation period as follows. At the termination of the experiment the gross gas exchange is directly measurable after addition of perchloric acid from the sidearm to the incubation medium, to release dissolved carbon dioxide. This corresponds to the $(O_2 - CO_2)$ volume. Potassium ferricyanide from the second side-arm is then tipped into the centre well which contains potassium permanganate; the reaction produces potassium hydroxide which absorbs the gaseous carbon dioxide and allows the nett oxygen utilisation to be measured after equilibrium is reached.

$$KMnO_4 + 3K_4\,Fe(CN)_6 + 2H_2O \longrightarrow MnO_2 + 3K_3\,Fe(CN_6) + 4KOH.$$

The difference between the nett and gross figures therefore is equal to the evolution of carbon dioxide (see Warburg & Krippahl, 1960). Two sets of data from this system, with analyses from the conventional centre well flasks which contained samples of the same trypanosome preparations, are shown in Table 12. No significant differences between the two single side-arm flasks $\pm$ KOH and the double side-arm flasks are apparent. However, this alone does not signify that carbon dioxide fixation is not involved in trypanosomal metabolism. When perchloric acid is added to a pair of flasks at the beginning of the experiment to enable the

TABLE 13

The effect of arsenicals on the utilisation of oxygen

Whole cells (approx. 0.2 mg N) or standard water-lysed preparations (approx. 1.0 mg N) were incubated in saline and MFM respectively in the presence of varying concentrations of the inhibitors. Oxygen uptake was measured over a 30 minute period, except in the presence of high concentrations of the inhibitors where inhibition increased with time. In these cases the initial rates of oxygen uptake were estimated. Each I_{50} value is from a series of at least five inhibitor concentrations and is given $\pm$ standard deviation. Other details as in Chapter 3.

		I_{50} (M)				
		Lysed cells			Whole cells	
Substrate:		Glucose	∝-oxoglutarate	Pyruvate	Glucose	∝-oxoglutarate
Strain	Inhibitor					
T.rhodesiense EATRO 173 77 - 80% thick	Melarsen oxide	$3.1\pm0.8\times10^{-5}$(4)	$2.0\pm0.8\times10^{-5}$(4)	$2.2\pm0.2\times10^{-5}$(5)	$5.0\pm0.9\times10^{-6}$(9)	$1.1\pm0.2\times10^{-5}$(4)
	Phenylarsenoxide	$4.1\pm0.7\times10^{-6}$(3)	$3.2\pm0.6\times10^{-6}$(6)	$4.0\pm0.8\times10^{-6}$(5)	$5.2\pm0.8\times10^{-7}$(8)	$8.8\pm1.6\times10^{-7}$(3)
T.brucei TREU 277 Monomorph	Melarsen oxide	$9.7\pm1.8\times10^{-6}$(3)	N.U.	N.U.	$2.1\pm0.9\times10^{-6}$(4)	N.U.

N.U. signifies substrate not utilised.

zero time concentrations of metabolites to be estimated, a considerable evolution of carbon dioxide occurs from the flasks containing KOH in the centre well. After approximately 8-10 minutes the gas is absorbed by the alkali, but this evolution does imply the presence of dissolved carbon dioxide in all the flasks during the experimental period. Hence the presence of KOH in the centre-wells of the respirometers does not remove all the available CO_2, due to the relatively slow evolution of the gas from neutral solution. The use of the standard manometric system on which this work is based will therefore not interfere with any metabolic CO_2 fixation reactions.

5) THE EFFECT OF TRIVALENT ARSENICALS ON THE METABOLISM OF T.RHODESIENSE.

The rapidity with which trypanosomes lost their motility and stopped utilising oxygen when suspended in high concentrations of melaminyl arsenicals, indicated that the action of these drugs is closely associated with the mechanism of energy production in the organism. The relative efficiencies of such a series of drugs may therefore be defined in terms of their respective abilities to inhibit the oxygen uptake of the organism, and the differential effects of a given drug on the oxygen utilisation from a series of substrates, may indicate the reaction involved in the inhibition. As will be seen in the following section on carbon balance studies with the arsenical drugs, an inhibition derived from experiments using only one concentration of inhibitor may be misleading, in so far as different reactions may be arrested by different drug concentrations. The I_{50} values for oxygen utilisation, as reported in Table 13, therefore are a measure of the overall effectiveness of the drugs, irrespective of the actual point of inhibition.

Phenylarsenoxide inhibits oxygen utilisation in all the systems investigated at concentrations an order of magnitude lower than those

required for equivalent effect by the melaminyl derivative. The I_{50} value for phenylarsenoxide in a whole cell system using glucose is about half that reported by Marshall (1948) who used a whole blood suspension of T.evansi. However, as differences in sensitivity occur even between strains of T.rhodesiense, this discrepancy is hardly surprising. Marshall also used a whole blood preparation of the trypanosomes, and although he showed that the blood elements contributed a negligible part to the oxygen uptake of his experimental material, no account was taken of arsenical uptake by the erythrocytes, a process which would have decreased the effective free concentration of inhibitor.

In all cases, a higher concentration of drug is required to produce 50% inhibition of the oxygen utilisation rate with a cell lysate, than that required with whole cell preparations. These differences were not as great as may have been expected on the basis of the data of Hawking (1937,1938), which showed a selective uptake of phenylarsenoxide by trypanosomes to give a cell to medium partition ratio of $5\text{-}10 \times 10^3$. This figure for arsenical accumulation, however, will be dependent upon the initial extracellular concentration of arsenical, and the free concentration of inhibitor in the glycolytic compartment of the cell may be much lower than the total intracellular concentration. The variation found between whole cells and lysates is also probably due in part to the liberation on lysis of non-specific arsenical binding sites, which will effectively lower the free drug concentration.

The melaminyl drug and its parent arsenoxide are powerful inhibitors of glucose supported oxygen uptake, and of the oxidative decarboxylations of pyruvate and α-OG. The similarity of the I_{50} values for the various substrates, appeared to indicate a substrate-independent point of action of the arsenicals. However the utilisation of oxygen with L-α-GP as the substrate, was uninhibited at 2×10^{-5} M-melarsen oxide and 3×10^{-6}

M-phenylarsenoxide. When the NAD^+-dependent L-α-GP dehydrogenase was assayed spectrophotometrically in the presence of these arsenicals, less than 15% inhibition was obtained at the above inhibitor concentrations. The two common steps in the pathway of electrons to oxygen, from glucose and the α-keto acids, i.e.

$$NADH + DHAP + H^+ \rightleftharpoons L\text{-}\alpha\text{-glycerophosphate} + NAD^+$$

and $$L\text{-}\alpha\text{-glycerophosphate} + \tfrac{1}{2}O_2 \rightleftharpoons DHAP + H_2O$$

appear therefore to be relatively insensitive to the arsenicals. From the data presented in Table 9 it may be seen that the utilisations of pyruvate and α-OG are confined to one-step oxidative decarboxylations, producing acetate and succinate respectively. As the reactions from NADH to oxygen are not markedly sensitive to the drugs, it would therefore appear that the oxidative decarboxylases themselves are target enzymes for the arsenicals. However, the monomorphic strain of T.brucei is sensitive to the arsenicals at the same drug levels as the pleomorphic organisms, indicating that these enzymes are not the primary trypanocidal targets.

Effects of melarsen oxide on the products of glucose metabolism by cell lysates.

The susceptibility of monomorphic T. rhodesiense to melarsen oxide, implies that any major therapeutic effect exerted by the drug on the catabolism of glucose by these organisms, is directed at some point prior to pyruvate in the degradative sequence. The productions of acetate and succinate from pyruvate, (reactions which the monomorphic strains have lost the capacity to carry out), are therefore not primarily connected with the trypanocidal activity of melarsen oxide, and these end-products of the glucose metabolism of the pleomorph were not

TABLE 14

Effects of melarsen oxide on glucose metabolism by cell lysates

Standard water-lysed preparations of T.rhodesiense EATRO 173 (approx. 1.5 mg N) were suspended in MFM with 25 µmoles in the presence of varying concentrations of melarsen oxide. Oxygen uptake was measured over a period of 30 minutes. Other details as described in Chapter 3.

Concentration of melarsen oxide ($\times 10^5$ M)	Substrate utilised		Metabolite produced			Metabolic ratios		
	Glucose (µmoles)	oxygen (µmoles)	Pyruvate (µmoles)	CO_2 (µmoles)	R.Q.	Pyruvate / Glucose	CO_2 / Glucose	O_2 / Glucose
Zero	12.69	11.41	8.69	6.74	0.59	0.68	0.53	0.90
1.15	12.14	9.56	9.77	4.11	0.43	0.81	0.34	0.79
2.30	8.54	7.24	8.85	2.50	0.34	0.96	0.29	0.85
3.45	5.01	4.69	3.76	2.06	0.44	0.75	0.40	0.94
Zero	10.13	9.22	7.02	5.48	0.54	0.70	0.54	0.91
1.15	9.09	7.45	7.54	4.12	0.55	0.83	0.45	0.83
2.30	6.78	5.24	6.67	2.41	0.46	0.98	0.35	0.77
3.45	4.89	4.15	3.51	1.50	0.35	0.72	0.31	0.85

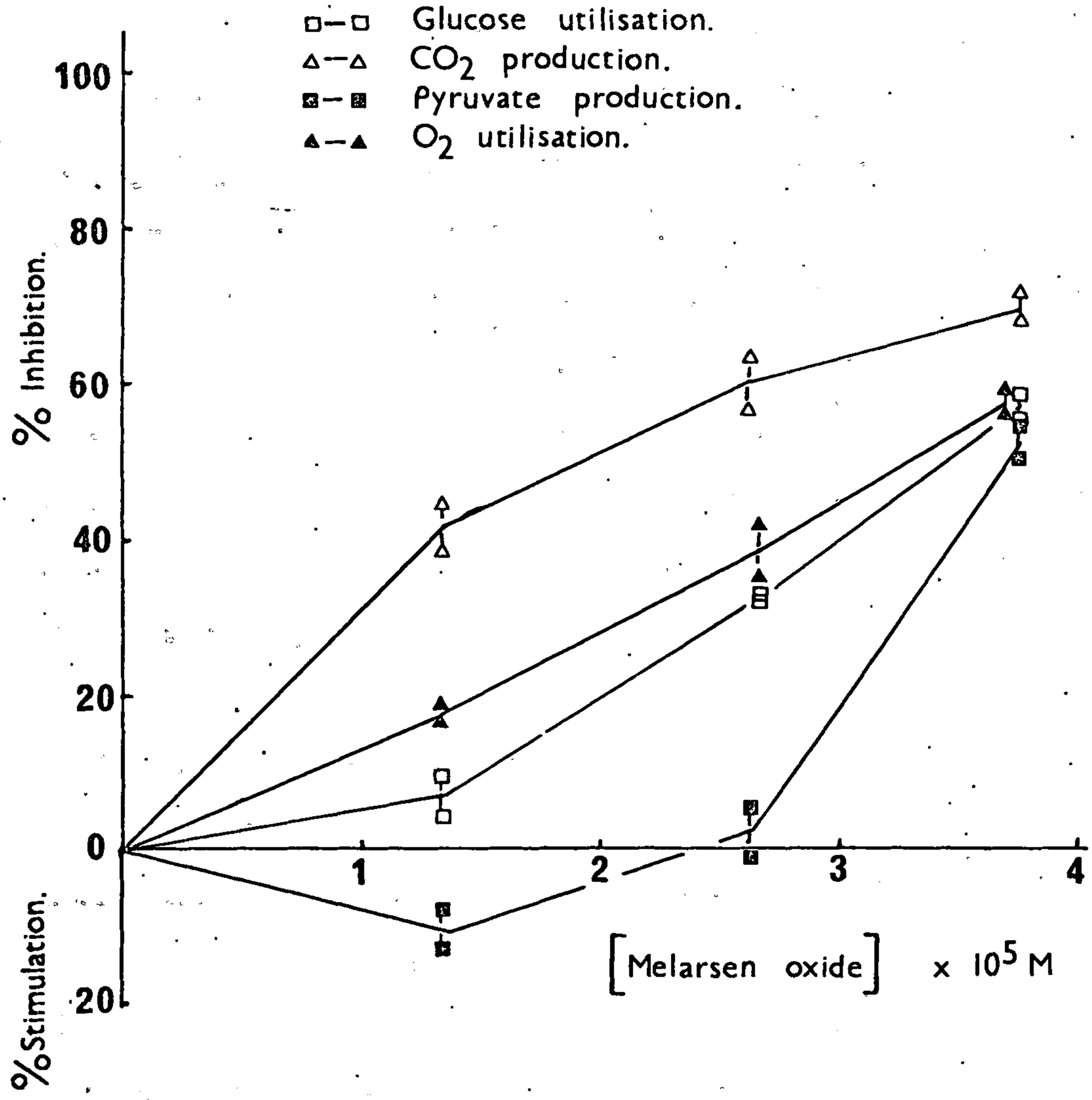


Fig. 8. Melarsen oxide inhibition of glucose metabolism by standard water-lysed preparations. The substrate utilisation and metabolite production data of Table 14 expressed as % inhibitions / stimulations.

estimated in the carbon balance studies using the arsenicals.

The inhibitory effects of melarsen oxide on pyruvate production from glucose by standard water-lysed T.rhodesiense EATRO 173, are shown in Table 14 and the same data are presented graphically in Figure 8.

At the lowest concentration of melarsen oxide (1.15×10^{-5} M Table 14) the gas exchange of the lysates is inhibited, whilst the nett amount of glucose utilised is not significantly altered. Accompanying this differential effect of the drug, is a stimulation of pyruvate production. The first point of action of the arsenical, therefore, is the pyruvate oxidase system. The decrease in oxygen utilisation is the result of the inhibition of two processes, the overall utilisation of glucose and the further metabolism of pyruvate. As previously stated, the oxygen uptake of cell lysates using L-α-GP is uninhibited at this concentration of arsenical. The decrease in glucose utilisation from Table 14 may be seen to be 0.55 μmole in experiment I, equivalent to a decrease in oxygen utilisation of 0.55 μmole in producing pyruvate. The gross decrease in oxygen uptake is 1.85 μmole, giving a nett decrease in uptake from pyruvate utilisation of (1.85 - 0.55) = 1.30 μmole oxygen. The parallel decrease in CO_2 production is 2.63 μmoles, giving an R.Q. of 2.02 for the decrease in gas exchange in the presence of the drug. Whereas this figure agrees well with the R.Q. obtained from lysates utilising exogenous pyruvate, the theoretical increase in pyruvate production accompanying the decrease in carbon dioxide production is 2.63 μmoles, as opposed to the 1.08 μmoles obtained experimentally. This "loss" of pyruvate carbon indicates that the drug is, in fact, partially inhibiting one of the reactions producing pyruvate, at the same time as it inhibits pyruvate utilisation. The inhibited reaction involved in the formation of pyruvate must be in

the part of the glycolytic sequence subsequent to the oxygen utilising step (i.e. the production of NADH by triose phosphate dehydrogenase), as otherwise the R.Q. of the "lost" gas exchange would be less than 2.0. A similar analysis of the second set of data from Table 14 shows an R.Q. of 1.86 for the nett decrease in pyruvate utilisation, and again a loss of approximately 40% in the theoretical increase in pyruvate formation.

Doubling the concentration of melarsen oxide further inhibits the carbon dioxide - oxygen exchange and a marked inhibition of glucose utilisation now accompanies the effects on gas exchange. Pyruvate production is still essentially unaffected. At this point, analysis of the arsenical effects along the lines indicated above does not give a clear-cut definition of the inhibitions. In both cases, the decrease in oxygen utilisation may be totally accounted for on the basis of the decrease in glucose uptake, and the basic weakness of these analyses becomes apparent, in that no account has been taken of the production of glycerol in the presence or absence of the inhibitor.

The production of 8.85 µmoles pyruvate (Expt.I, 2.30×10^{-5} M-melarsen oxide) requires the utilisation of 4.43 µmoles oxygen. Assuming that all the CO_2 production is from the decarboxylation of pyruvate to produce acetate, another 1.25 µmoles of O_2 are required at this step, giving a maximal total requirement of 5.68 µmoles oxygen, compared to the observed utilisation of 7.24 µmoles. Again, assuming that no other routes of glucose metabolism are being used in these lysates, a buildup of carbon between 3-phosphoglycerate and phosphoenol-pyruvate is indicated.

Further increase in the drug concentration produces a multipoint inhibition. Hexokinase becomes inhibited markedly as seen from the drop in utilisation of substrate, and the data become uninterpretable in terms of specific inhibitory effects. This inhibition of hexokinase

TABLE 15

Effects of melarsen oxide on glucose metabolism by whole cells: 1) Inhibitor titration

Whole cells of T.rhodesiense EATRO 173 (approx. 0.3 mg N) were suspended in saline and incubated under the same conditions as those described in Table 14. All other experimental conditions are as described in Chapter 3. Pleomorphic composition 81%, 81%, 76%, 79% SS.

Concentration of melarsen oxide ($\times 10^6$ M)	Substrate utilised (µmoles)		Metabolite produced (µmoles)	
	Glucose	Oxygen	Pyruvate	CO_2
Zero	8.41	8.99	10.43	3.62
1	6.06	6.18	6.19	2.79
3	5.04	5.24	4.54	2.32
5	4.54	4.90	3.27	1.91
Zero	7.62	7.77	9.91	3.43
1	5.33	5.91	5.15	2.51
3	4.42	4.94	3.70	2.16
5	3.65	3.50	2.63	1.68
Zero	9.04	9.94	11.84	4.34
2	6.15	7.01	6.16	3.14
4	5.24	5.55	4.14	2.41
6	4.43	4.58	3.10	1.90
Zero	8.04	8.05	9.81	3.62
0.1	7.72	7.73	8.34	3.51

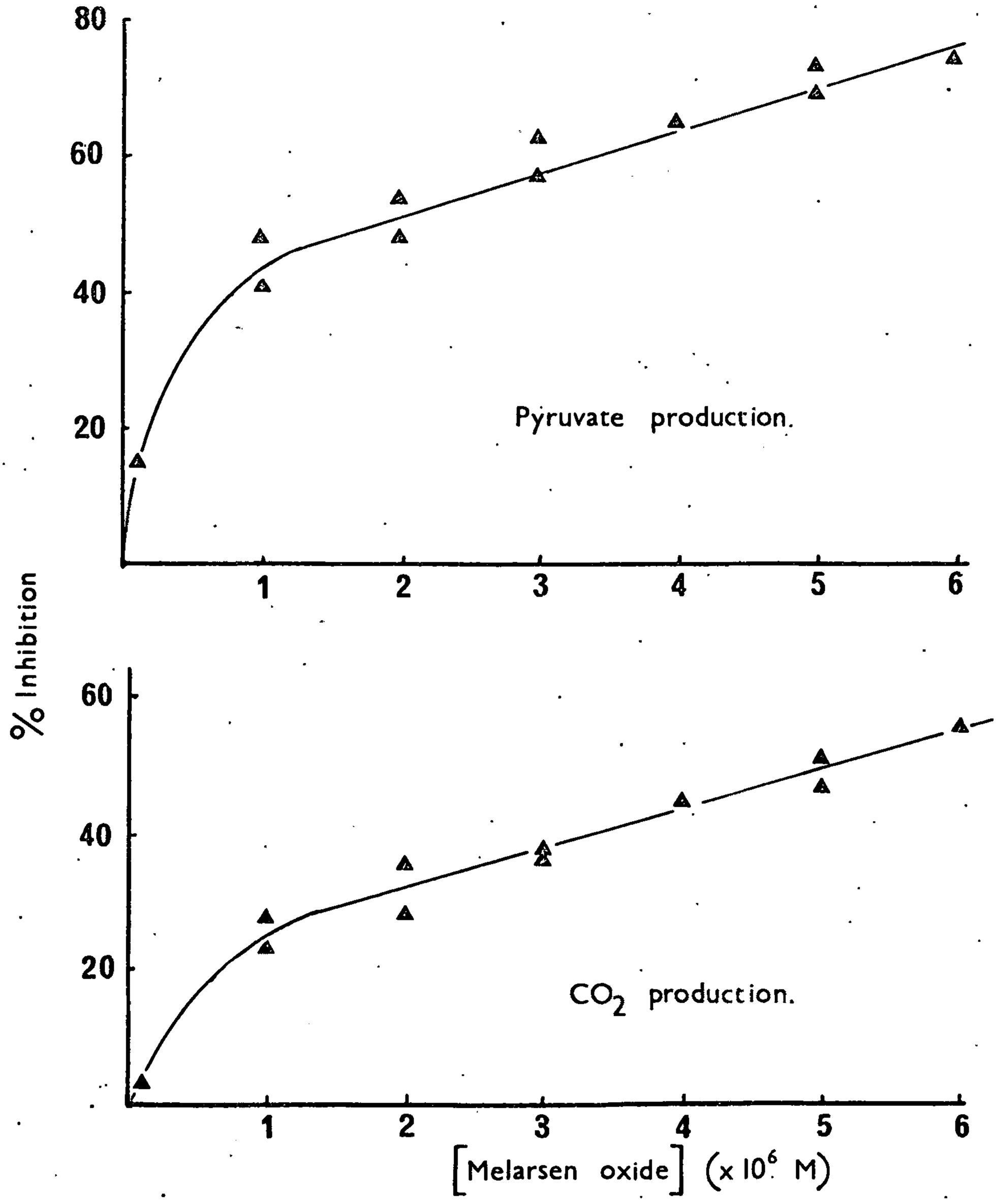


Fig. 9. Melarsen oxide inhibition of glucose metabolism by whole cell preparations.

b). The production of pyruvate and CO_2.

The data of Table 15 expressed as % inhibitions.

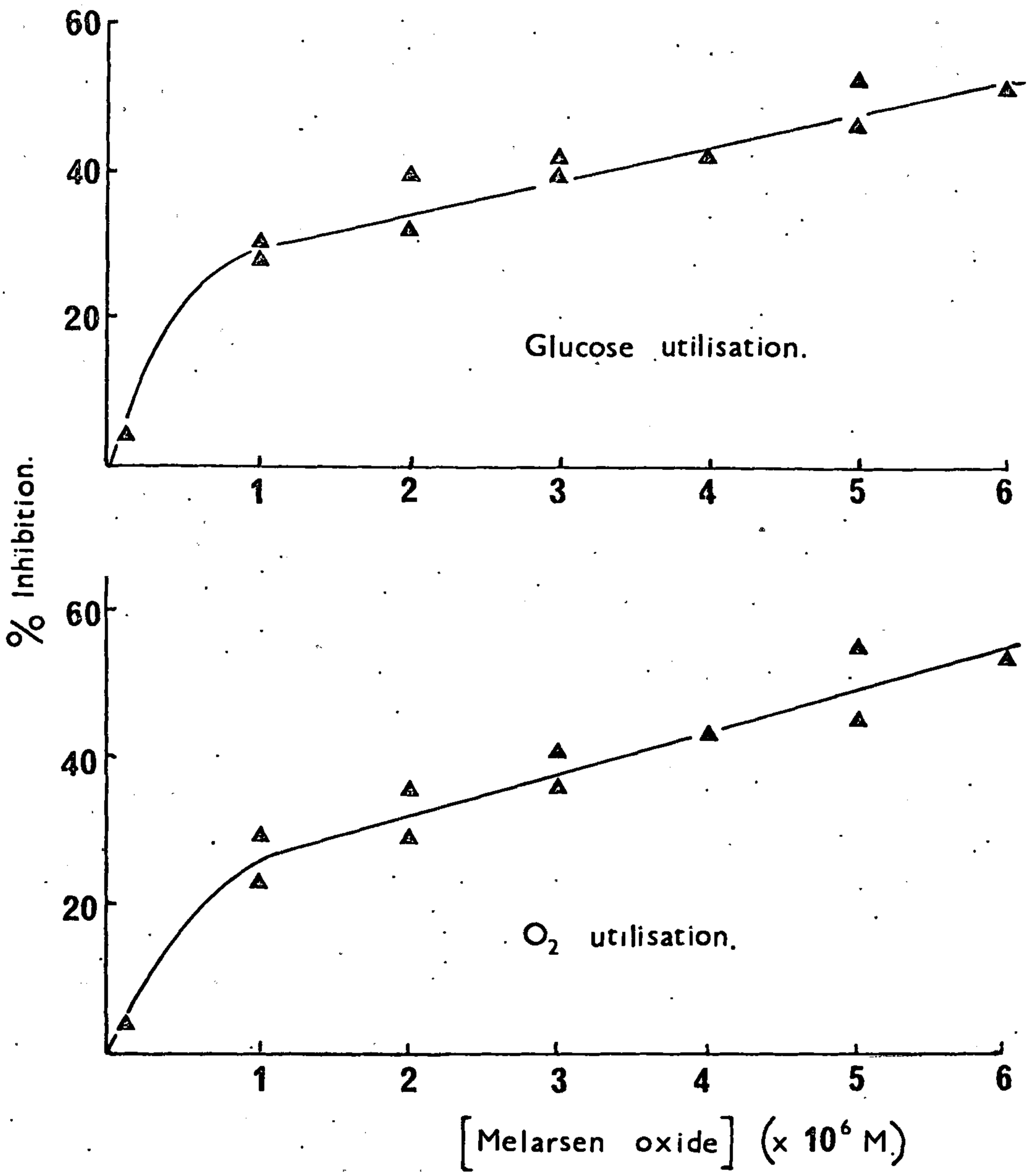


Fig. 9. Melarsen oxide inhibition of glucose metabolism by whole cell preparations.

a). The utilisation of glucose and oxygen.

The data of Table 15 expressed as % inhibitions.

may, however, be due to a decrease in the availability of ATP. The pattern of metabolic changes obtained by incubation of whole cells of the pleomorphic trypanosomes with melarsen oxide was found to be quite different to the lysate effects, indicating that extrapolation of the results obtained with cell lysates to account for the in vivo effects leads to difficulties in interpretation. No further experimentation was therefore carried out on the lysed material.

Effects of melarsen oxide on the products of glucose metabolism by whole cells.

The oxidative decarboxylation of glycolytic pyruvate appears to be the primary enzymic point of attack of melarsen oxide in the water-lysed system, in which all semblance of compartmentation will have been lost. No indication of a similar preference for this system could be found, however, in the intact organisms.

Table 15 contains the results of four series of experiments, the experimental variable being the concentration of melarsen oxide present, and these data are plotted in terms of percentage inhibition versus drug concentration in Figure 9 (a and b). These figures may be compared with Figure 8 which contains the analogous data pertaining to the lysed cell preparations; in this present case, the coincidence of the inhibitions of glucose and oxygen utilisation, and of the production of carbon dioxide, necessitates the separation of the graphical representations for reasons of clarity. All three of these parameters have I_{50} values in the region of $5 - 6 \times 10^{-6}$ M-melarsen oxide: The production of pyruvate is more sensitive to this drug, with an I_{50} value in the range $1 - 2 \times 10^{-6}$ M-melarsen oxide. No inflections are to be found in any of these experiments, analogous to that obtained for

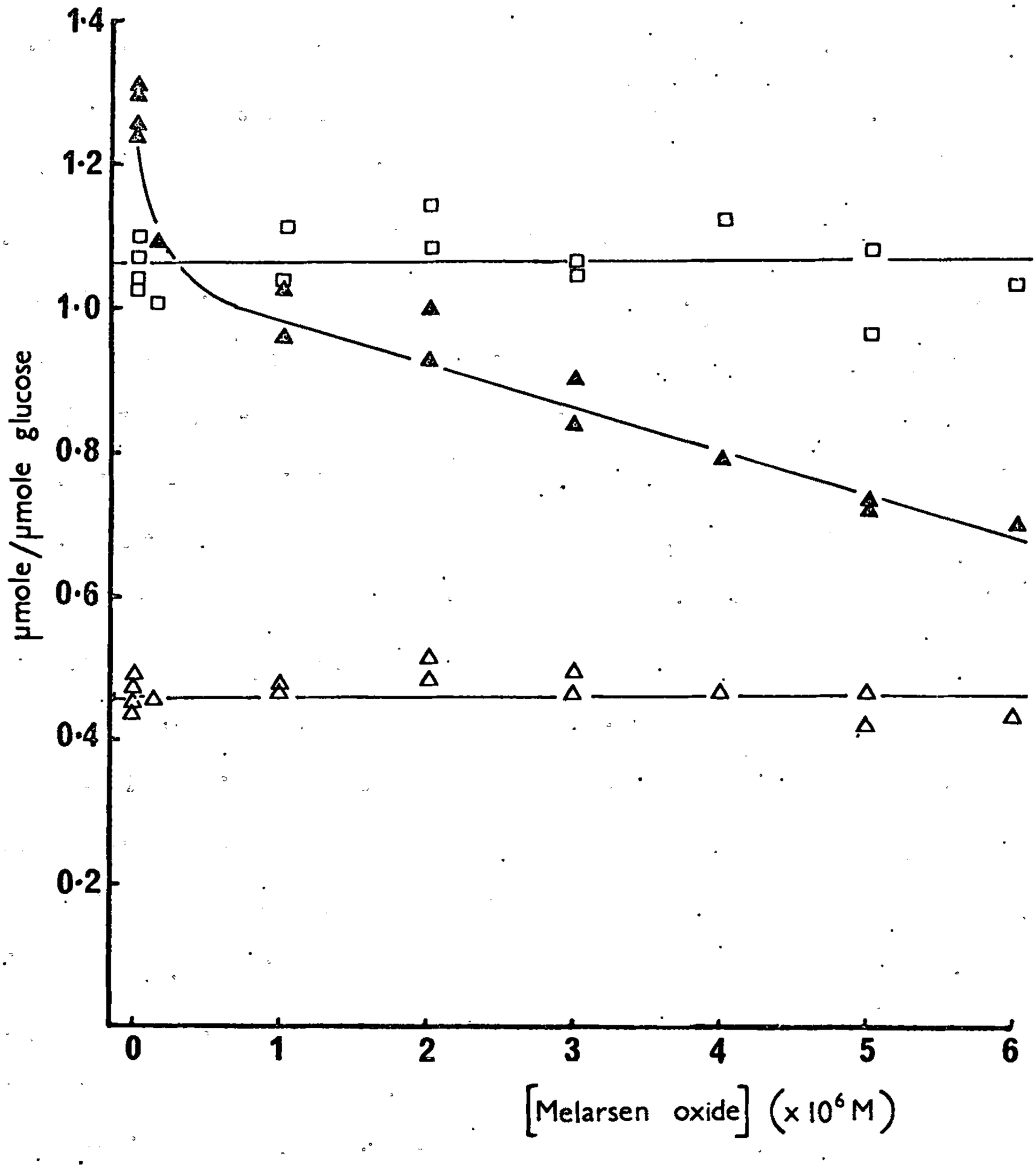


Fig. 10. Melarsen oxide inhibition of glucose metabolism by whole cell preparations. The data of Table 15 expressed as molar ratios with respect to the utilisation of glucose.
□ = oxygen utilisation. ▲ = pyruvate production.
△ = CO_2 production.

pyruvate production by cell lysates at different arsenical concentrations. Stimulation of the production of this end-product, theoretically obtained by a specific inhibition of the pyruvate oxidase system, could not be demonstrated in the intact cells at any concentration of melarsen oxide. The lowest concentration of inhibitor used experimentally, (10^{-7} M), produced an inhibition of 15% of the production of pyruvate, simultaneously having a negligible effect ($\leqslant$ 4% inhibition) on the utilisation of glucose and oxygen or the production of carbon dioxide. Under in vivo conditions the primary inhibition by this arsenical would appear to be prior to the formation of pyruvate, in the glycolytic chain. By a similar logic to that used in the interpretation of the results obtained with lysed cell preparations, this inhibition cannot be between glucose--6--phosphate and the triose phosphates, or a decrease in the ratio of oxygen utilised to glucose utilised would be apparent; Figure 10, which contains the data of Table 15 expressed in this manner, shows this ratio to be more or less constant over the range of inhibitor concentrations used. A build up of an intermediate of the glycolytic pathway between 1:3 diphosphoglyceric acid and PEP is therefore expected. At low levels of inhibitor (10^{-6} M,) the utilisation of glucose is itself inhibited, indicating that hexokinase is sensitive to melarsen oxide. (This inhibition may again be due to a decrease in the availability of ATP due to the primary inhibition of pyruvate kinase.) This is borne out by the parallel nature of the inhibitions of glucose and oxygen consumption and carbon dioxide production; assuming that the evolution of carbon dioxide is at least in part due to the production of acetate, it would appear that either this system is uninhibited by melarsen oxide in vivo or the drug cannot permeate into the cellular compartment containing the oxidase system. The highest concentration of drug utilised in the whole cell experiments is 6×10^{-6} M. At this concentration,

TABLE 16

Effects of melarsen oxide on glucose metabolism by whole cells: 2) PEP accumulation

Experimental details as in Table 15, with melarsen oxide at 2×10^{-6} M. Pleomorphic composition 78% SS. Metabolite estimations carried out as in Chapter 3. Experimental duration 45 minutes.

	Standard		+ Melarsen oxide		
	µmoles	% Glucose carbon	µmoles	% Glucose carbon	% Inhibition
Glucose utilisation	8.61	100	5.34	100	38
Oxygen utilisation	8.95	-	5.77	-	35
Pyruvate production	10.85	63	4.97	47	46
CO_2 production	3.96	8	2.56	8	36
PEP production	approx. 0.35	approx. 2	1.05	10	300% stimulation

the utilisation of pyruvate by cell lysates as measured by the utilisation of oxygen in the presence of pyruvate as substrate, is 18% sensitive to melarsen oxide, and hence the latter of those alternatives is preferable. When the concentration of the drug by the trypanosomal cell is taken into consideration, this argument is reinforced.

By analogy with the inhibition of hexokinase by melarsen oxide, two enzymes in the second half of the glycolytic sequence appeared as possible alternatives for the primary inhibitory point by which a decrease in pyruvate yield is possible, without a concomitant decrease in oxygen uptake or glucose utilisation. These are the two kinases, catalysing the production of 3-phosphoglyceric acid from 1,3-diphosphoglycerate and of pyruvate from PEP. Of these enzymes, the latter appeared the most promising alternative, as a buildup of 1,3-diphosphoglycerate accompanying an inhibition of phosphoglyceric acid kinase, may have been expected to affect the uptake of oxygen via a product effect upon the production of NADH by glyceraldehyde-3-phosphate dehydrogenase.

Accordingly, estimations of PEP were carried out on normal and inhibited whole cell preparations, the results of which may be found in Table 16. Approximately 60% of the glucose carbon no longer found as pyruvate in the presence of the drug, is accounted for as a buildup of PEP, leading to the conclusion that pyruvate kinase is the focal point of arsenical action in this part of the glycolytic sequence.

The second part of this work is accordingly devoted to a comparative study of the pyruvate kinases of trypanosomal and of mammalian systems, and the effects thereon of melarsen oxide.

CHAPTER 5

EXPERIMENTAL AND RESULTS -

PYRUVATE KINASE

1) CHOICE OF MATERIAL AND ASSAY CONSTITUENTS.

The preferred assay system for pyruvate kinase, that of Bücher and Pfleiderer (1962), is dependent upon the utilisation of NADH by LDH in the presence of pyruvate produced by the kinase, and is followed spectrophotometrically by the decrease in absorbance at 340 nm. The use of freshly lysed trypanosomal material was investigated as a starting point for work on PK, but was discarded. The relatively high rate of NADH oxidation occurring in the presence of water-lysed material without addition of exogenous substrate, the irreproducibility of this rate between different lysed samples, the time entailed in the preparation of the organisms free from blood elements, and the finding that PK activity in fresh lysates was unstable, were all factors which obviated their use.

Acetone powders of this material likewise were discarded as an experimental source of the enzyme, as recoveries of activity from such powders were exceptionally low. Lyophilisation, however, yielded the trypanosomal material in a convenient and active state as described below.

Effects of lyophilisation on pyruvate kinase.

Estimates of the PK activity present in fresh lysates could be obtained after correction for the endogenous rate of oxidation of NADH, although the instability of the enzyme made these estimates to a certain extent unreliable. Comparison of this activity with the rates obtained from lyophilised material, showed that from 55% to 75% of the total activity was retained on freeze-drying. Apart from subjecting the trypanosomal material to lyophilisation as soon as possible after lysis of the cells, no attempts were made to improve these yields. Any

TABLE 17

Pyruvate kinase activity and physico-chemical properties of T.rhodesiense and T.brucei

Trypanosomes were sedimented for 10 minutes at 1000g to give the packed cell volume (PCV) and lyophilised. Protein and total Nitrogen (N) estimated as in Chapter 3. PK activity of the lyophilised material assayed in the standard assay system, with 5.0 µmol PEP and 1.25 µmol ADP.

	Batch	PCV (ml)	Dry weight (mg)	Protein (mg)	Total N (mg)	PK activity (µmol/min/mg protein)
T.brucei TREU 277	1	1.1	94	66	9.2	1.04
	2	0.8	71	43	5.3	0.74
	3	0.9	80	52	6.7	0.81
T.rhodesiense EATRO 173	1	0.6	55	35	4.4	0.91
	2	0.8	72	45	5.6	0.86

batch of material with a specific activity of less than 0.5 /umole / minute /mg protein was discarded.

In the lyophilised state, the pyruvate kinase activity was found to decrease gradually on storage at 2°C, but this deterioration was practically eliminated by storage in vacuo at this temperature. Batches of material stored in this fashion for up to two years' have been found to retain over 80% of their activity. Prior to use, each individual batch of lyophilised material was assayed for lactate dehydrogenase activity, and only those showing a complete absence of this enzyme (indicating purity from blood element contamination) were used for investigation of the trypanosomal pyruvate kinase.

Table 17 indicates the relationship between the activity of this enzyme and the characteristics of the lyophilised material from pleomorphic and monomorphic strains. The rate of utilisation of PEP ranges from 0.74 to 1.04 /umole/min/mg protein, whereas the in vivo rate of glucose utilisation is of the order of 1.0 /umole/min/mg protein i.e. equivalent to 2 /umole PEP/min/mg protein. The losses of activity on freeze-drying only raise the total activity to approximately 1.4-1.9 /umole/min/mg protein assuming the maximum loss of 45% of the activity, which still does not account for the observed in vivo turnover rate. However, two aspects of the properties of this enzyme, namely the activation by thiol compounds and by fructose 1,6 diphosphate, indicate that the in vivo activity is in fact sufficient to account for the total metabolism of glucose to pyruvate.

Standard assay of trypanosome pyruvate kinase.

i) Buffer

Early experiments using the phosphate buffer system of Bücher and Pfleiderer (1962) gave values of PK activity of a very minimal and variable nature. It was found that the phosphate anion was markedly

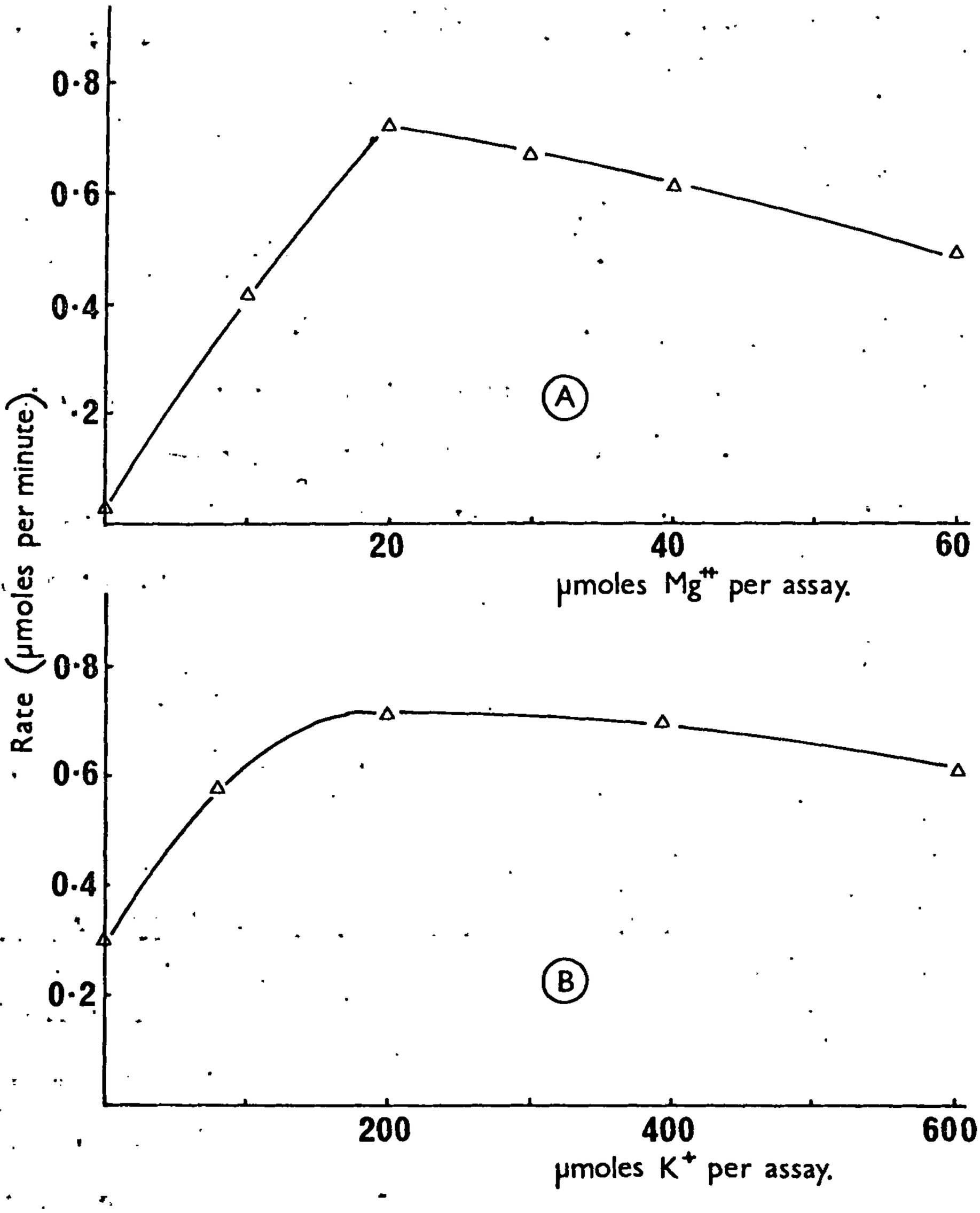


Fig. 11. The effect of cation concentration on the activity of unpurified pyruvate kinase. The enzyme source was lyophilised T. rhodesiense EATRO 173 resuspended in water at 2.0 mg/ml. Aliquots (0.1 ml) were assayed at the indicated cation concentrations with A) 200 μmoles KCl per assay and B) 20 μmoles $MgSO_4$ per assay. All other assay conditions were standard, as described in Chapter 3. Reactions were initiated by addition of enzyme.

inhibitory to this enzyme, and a five-fold increase in activity at pH 7.2 was obtained on substitution of a triethanolamine buffer at this pH.

ii) Cation requirements.

The effects of varying K^+ or Mg^{2+} ion concentration at a fixed concentration of the other cation are shown in Figure 11. These data are from the last in a series of experiments in which the concentrations of both cations were varied both independently and together to find the optimum conditions. As the sole purpose of these experiments was to define the optimal conditions of assay, no account of the potassium ion content of the buffer has been taken.

With the exception of these two parameters, the assay system used is standard. All other details are to be found in Chapter 3..

2) STABILITY AND STABILISATION OF TRYPANOSOME PYRUVATE KINASE.

After resuspension of freeze-dried trypanosomes in water, a large decrease in activity is found in the first 24 hours storage, either at 17°C or at 2°C. The extent of the loss in activity is dependent upon the concentration of the resuspended material, but is very variable between batches of lyophilised material. The enzyme, in simple aqueous solution, was also found to possess a negative temperature coefficient of activity, a property known to be associated with hydrophobic enzyme proteins.

The results of experiments aimed at the stabilisation of PK are found in Table 18. ADP and PEP were used due to their function as cosubstrates of the reaction, Mg^{2+} and K^+ ions as activating cofactors, BSA in an attempt to mimic a further increase in the concentration of the lyophilised material and glycerol to provide a more hydrophobic environment. Ammonium sulphate, EDTA, and the thiol compound DTT are

TABLE 18

Stabilisation of trypanosome pyruvate kinase

Lyophilised T.brucei TREU 277 was resuspended in water and aliquots of the resuspension were fortified as indicated. Samples were stored in snap-top bottles, and samples were assayed for PK activity at the intervals shown, by the standard procedure. All incubations were in a final volume of 3 ml and were at pH = 7.2. Activities are expressed as percentages of activity remaining after storage, 100% in each case being the activity of that sample at zero time.

	% activity remaining					
	17°C			2°C		
Storage conditions	12h	24h	48h	12h	24h	48h
1) H_2O at 1.0 mg/ml	48	26	10	40	20	zero
2) H_2O at 5.0 mg/ml	69	50	38	49	36	28
3) 2) + 66.7 mM-KCl	52	26	12	43	25	zero
4) 2) + 6.7 mM-$MgSO_4$	58	29	11	47	21	4
5) 2) + 0.1 M-TEA	51	31	15	40	21	4
6) 2) + 10mM-EDTA	14	zero	-	10	zero	-
7) 2) + 1.67 mM-PEP	11	zero	-	11	zero	-
8) 2) + 0.42 mM-ADP	21	8	-	16	2	-
9) 2) + 10 mg/ml BSA	40	34	18	40	28	16
10) 2) + 25% $^{v}/_{v}$ glycerol	70	50	36	77	65	50
11) 2) + 0.1 mM DTT	78	56	42	85	69	59
12) 2) + 70% $^{w}/_{v}$ $(NH_4)_2SO_4$	49	28	19	41	24	6

- denotes sample not assayed.

three known enzyme stabilising agents. The contamination of the incubations was minimised either by the inclusion of a crystal of thymol, or by use of a toluene overlay. No inhibition of activity was found with either of these bacteriostatic agents.

Of the above reagents, only glycerol and DTT gave any protection against inactivation, and both these agents were more effective under refrigerated conditions than at room temperature. Throughout subsequent work on the purification of the kinase, a solvent system of 25% ($^{v}/v$) glycerol / 10^{-3} M-DTT was used, with 0.1 M-tris titrated to pH 7.2 with KOH/HCl. Since these conditions were defined, it is of interest to note that two authors have recommended similar protective media for stabilisation of the highly unstable liver type L pyruvate kinase isoenzyme: Tanaka et al., (1967a) reported that addition of 0.5 mM-ADP, 0.5 mM-PEP and 10^{-3} M-DTT stabilised this enzyme, and Rozengurt et al. (1969) utilised 10^{-3} M-DTT, 0.25 M-sucrose and 0.15 M-KCl to effect stabilisation at $4^{\circ}C$. In the case of the trypanosome enzyme, it may be seen that both ADP and PEP actually decrease the stability of the enzyme on storage, as do EDTA and dilution of the resuspended lyophilised material. The effect of glycerol is analogous to that of sucrose in the system of Rozengurt et al., (1969) in decreasing solvent interaction with the hydrophobic protein in aqueous solution. These similarities are of note in so far as the trypanosome enzyme has been shown to have many kinetic properties in common with this isoenzyme of mammalian (host) liver.

Although triethanolamine is used throughout this work as the buffer for enzyme assay, this material was found to interfere markedly with the estimation of protein by the method of Lowry et al., (1959) giving a very intense blue colour with the reagent.† A tris buffer system was therefore used during any experiments in which protein determination

† This interference is probably due to the formation of a complex between Cu^{++} ions and triethanolamine in the alkaline assay solution.

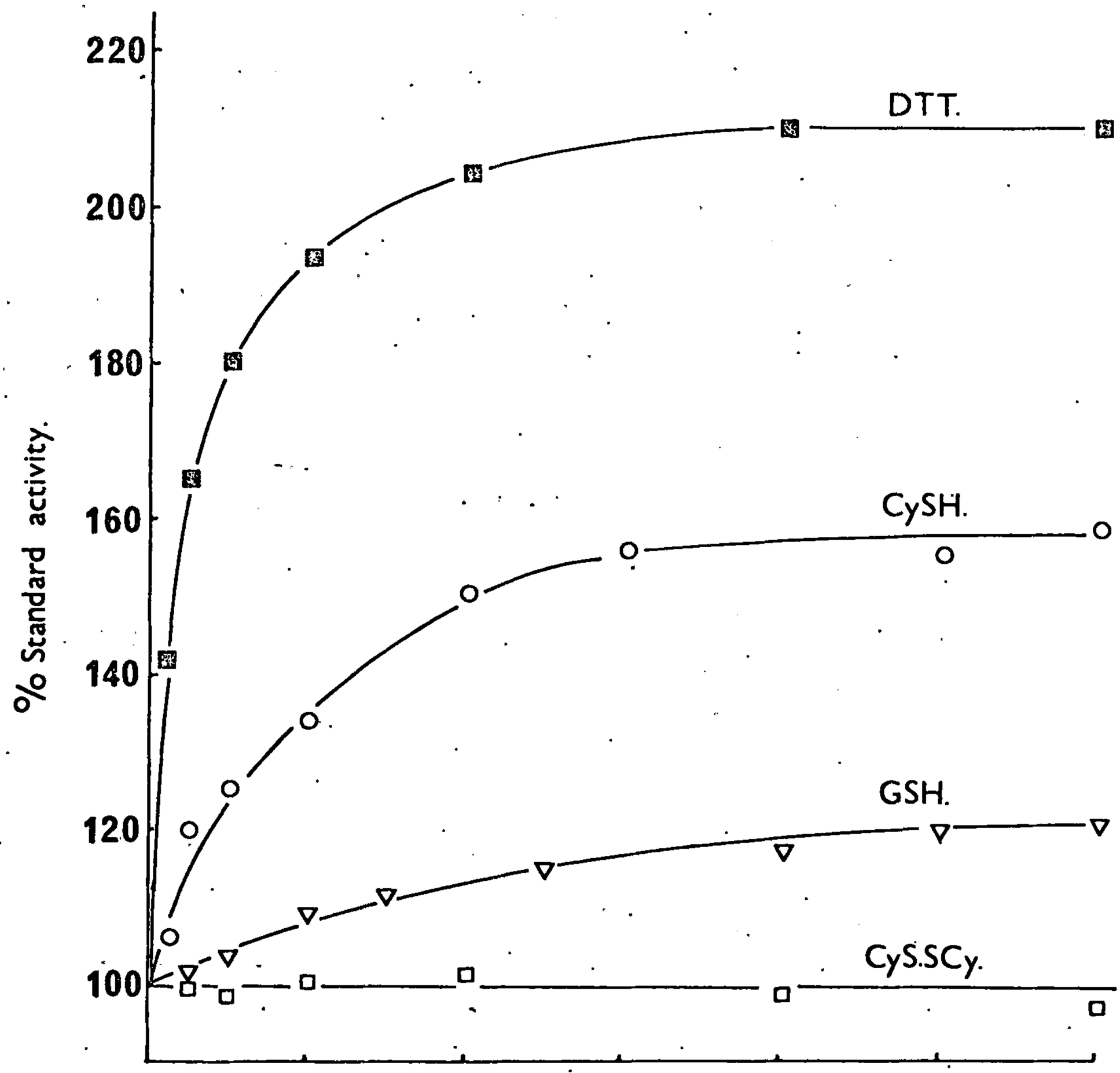


Fig. 12. Thiol activation of unpurified pyruvate kinase. Lyophilised, resuspended T. brucei TREU 277 (0.16 mg/assay) was preincubated in the standard assay medium with and without the thiol compounds as indicated, for 3 minutes at 25°C. Reactions were initiated by addition of ADP (1.25 µmol/assay) and PEP (5.00 µmol/assay). Results are expressed as % of the rates obtained in the absence of exogenous thiol.

was required. Tris itself interferes with this determinative method (Gellert et al., 1959) as does glycerol (Zishka & Nishimura, 1970) but these interferences were found to behave in a reproducible manner, and could be controlled.

Effects of thiols on unpurified pyruvate kinase.

During the attempts at stabilisation of the trypanosomal PK it was found that DTT, as well as stabilising the enzyme, had a marked stimulatory effect. This effect, along with the actions of two other reduced thiols, glutathione and cysteine, is illustrated in Figure 12. The oxidised form of one of these thiols, cystine, is included to show that this is an effect attributable to the reduced -SH group. Oxidised glutathione similarly produced no activation. Variable results are obtained unless the thiols, after dissolution in degassed distilled water, are kept under a layer of petroleum ether. The total activation by DTT (10^{-3} M) observed with different batches of lyophilised material, ranged from 84% to 128%, presumably due to variation in the oxidation of the samples during lysis and the freeze-drying process. Although no experiments were performed on the variation of stimulation with duration of storage, the increased stability of the preparations when stored in vacuo may be ascribed to elimination of the oxidation process. In a similar fashion the instability of resuspended lyophilised material is probably in part due to oxidation.

The thiol concentration required for 50% maximal stimulation was very much lower in the case of the dithiol, DTT (1.5×10^{-5} M) than with the monothiols (GSH : 3×10^{-3} M; CySH : 3×10^{-3} M) and the total extent of activation was also higher with the dithiol reagent. Increasing the concentration of cysteine to 0.1 M, and prolonging the incubation time did not increase the activation above 70%: this marked preference

of the enzyme for DTT appears also in other enzyme systems (Threonine deaminase, Burns & Zarlengo, 1968; Pyruvate kinase of E.coli, Maeba & Sanwal, 1968a). The work of the latter authors showed that E.coli PK is inactivated by dialysis and reactivated by DTT. Ultracentrifugal studies showed that, dependent upon the reduction of thiol groupings, this reversible inactivation appears to be an association/dissociation phenomenon. It has not been possible to determine whether an analogous dimerisation occurs on treatment of the trypanosome enzyme with the dithiol, due to the low yield and nature of the purified enzyme. However, that the enzyme requires at least one thiol group in the reduced form to retain activity, appears a plausible hypothesis on the basis of this action of the thiol reagents; as such PK appeared as a suitable target for inactivation by the arsenical drugs which (as far as is known) are specific thiol inactivating agents.

3) PROPERTIES OF TRYPANOSOME PK IN UNPURIFIED LYOPHILISED MATERIAL

Characterisation of an enzyme in an unpurified state is obviously desirable in so far as any purification procedure is liable to remove the protein even further from its natural environment, and may induce properties which are normally absent. In the case of trypanosome PK, as far as may be judged the process of purification does not markedly affect its kinetic properties, and so only a few properties of the unfractionated enzyme will be described in this section, as purification in this instance was later found to be obligatory for kinetic analysis.

It was found that prolonged storage of resuspended material in glycerol /DTT resulted in a gradual and variable decline in the sensitivity of the PK to melarsen oxide. The use of DTT in this fashion is also impractical, as the presence of the thiol would effectively lower the concentration of arsenical available for reaction with

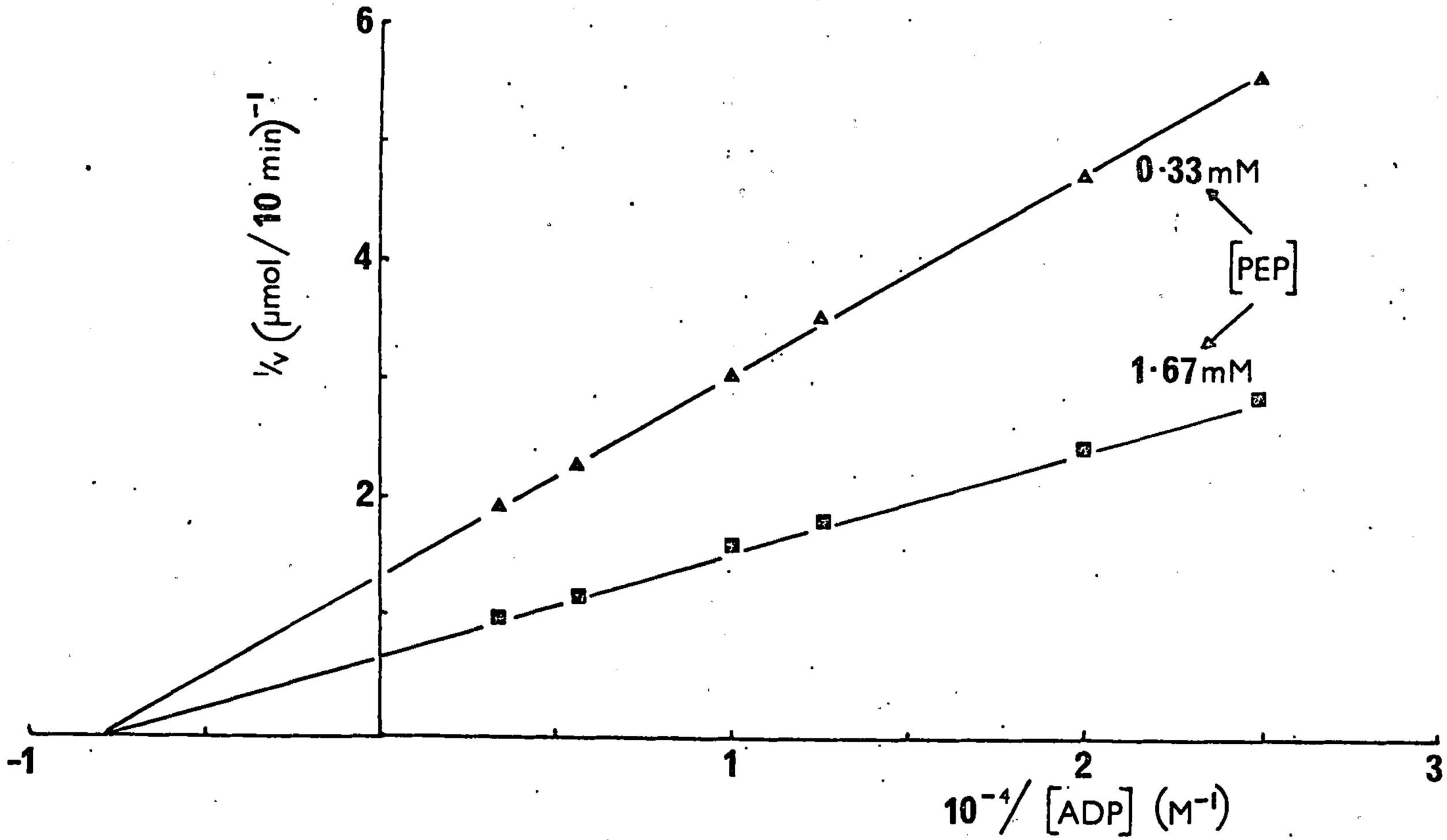


Fig. 13. The effect of ADP concentration on the activity of unpurified pyruvate kinase. Lyophilised, resuspended T. brucei TREU 277 (0.16 mg/assay) was preincubated in the standard assay medium for 3 minutes at 25°C. Reactions were initiated by addition of ADP and PEP as indicated. Initial rates were measured and Lineweaver-Burk plots derived as described in Chapter 3.

the enzyme, by formation of the cyclic thioarsinite. To avoid these complications all results obtained on the crude material were produced with lyophilised trypanosomes resuspended in double-distilled water, and all preparations were discarded not more than four hours after resuspension, during which time they were routinely stored in ice. Over this period, whereas the decrease in activity was measurable (12-20%) and was in all cases corrected for, the kinetic and inhibition characteristics of the enzyme which were reliably estimable, appeared to be unchanged. For purposes of correction, the standard activity with 5 /umoles PEP and 1.25 /umoles ADP was measured at least every fifteen minutes and all results were adjusted to allow for the percentage decrease in activity of this standard system.

Effect of ADP concentration.

The kinetic data of Melchior (1965) and Ibsen et al., (1968) suggested that the monomagnesium salt of ADP^{3-}, and not the free coenzyme, is the specific cofactor in the pyruvate kinase reaction, although evidence is to be found (Mildvan & Cohn, 1966) that free Mn^{2+} and free ADP^{3-} do bind to the enzyme. Under the conditions of assay used in this work, effectively all the ADP present is in the form of $MgADP^{-}$. Using the value of 2×10^3 (O'Sullivan & Perrin, 1964) for the estimated affinity constant for $MgADP^{-}$, it is possible to calculate the concentrations of free and combined ADP in solution at various concentrations of Mg^{2+}. The range of concentrations of ADP used to determine the K_M value for this coenzyme are such that at all times the concentration of free ADP^{3-} is negligible, and the kinetic constant derived is descriptive of the $MgADP^{-}$ complex.

Figure 13 illustrates double reciprocal plots of the enzymic rate versus ADP ($MgADP^{-}$) concentration, at two concentrations of PEP.

Calculation of the extrapolated K_M values gave $1.31 \pm 0.04 \times 10^{-4}$ M-ADP at 1.0 /umole PEP per assay and $1.30 \pm .03 \times 10^{-4}$ M-ADP at 5.0 /umole PEP per assay. The range of figures for this parameter obtained from many estimations on different batches of lyophilised material was from $1.21 - 1.39 \times 10^{-4}$ M-ADP, with a mean of $1.30 \pm 0.16 \times 10^{-4}$ M-ADP, (Eleven determinations).

As a check on the methodology and for comparative purposes, the K_M value for ADP was estimated under identical conditions using a commercial preparation of rabbit muscle PK. Three separate estimations gave a mean value for K_M ADP as $2.06 \pm 0.07 \times 10^{-4}$ M-ADP, a figure close to that of Reynard et al., (1961) of 2.1×10^{-4} M-ADP.

Thus in a highly impure enzyme source, it may be seen that the affinity of the trypanosome enzyme for ADP in the $MgADP^-$ form is slightly higher than that of the muscle enzyme, and is independent of the concentration of PEP over the range 0.33 - 1.67 mM.

Effect of PEP concentration

Whereas the effect of ADP concentration on the reaction rate was reproducible from batch to batch of the lyophilised material, completely irreproducible results were obtained when the effect of variation of the concentration of PEP was studied. Double reciprocal graphical representations of velocity versus PEP concentration gave curves which varied from an approach to linearity to a marked upward concavity. Hill plots of these data varied in slope from n = 1.3 to n = 1.8, and at the time no explanation of this variability was found. Later investigations on the purified enzyme led to the conclusion that the presence of varying amounts of FDP in the lyophilised material may account for these anomalous results (vide infra). Due to these inconsistencies inherent in the use of the crude enzyme, no data will be presented for kinetic parameters

relating to PEP utilisation, and the description of other work carried out on unpurified enzyme preparations will be confined to some preliminary investigations of inhibitor action, the interaction of the arsenicals with the lyophilised material, and the presence of interfering enzymes.

Interference by contaminating enzymes. a) enzymes utilising PEP

The most likely source of error as far as the utilisation of PEP is concerned, is by reversal of part of the glycolytic sequence which produces PEP. The presence of both phosphopyruvate hydratase (E.C. 4.2.1.11) and of phosphoglycerate 2,3 phosphomutase (E.C. 5.4.2.1) was demonstrated in the lyophilised material, by the oxidation of NADH in the presence of LDH and 2-PGA or 3-PGA respectively. When quantitative carbon-balance studies were carried out on the utilisation of PEP by lyophilised material, the following data were obtained:

Enzyme batch	/umoles PEP utilised	/umoles Pyruvate formed	% yield
1	1.33	1.14	86%
2	1.21	1.10	91%
3	1.14	1.07	94%

With a measure of inter-batch variation, PEP of the order of 5-15% is being utilised by some pathway other than in the formation of pyruvate. When these enzyme preparations were incubated with pyruvate under identical conditions, recoveries of between 98% and 101% were obtained. Thus the loss of PEP carbon is not by further utilisation of the product.

b) Other enzymes

Qualitative estimation of phospbate production on incubation of resuspended lyophilised material with ATP or FDP showed both ATPase and FDPase (or a non-specific phosphomonoesterase) to be present in the

TABLE 19

Effect of inhibitors on crude trypanosome and purified rabbit muscle pyruvate kinases

Enzyme (approx. 0.2 mg dry weight trypanosomes, or 0.1 I.U. commercial muscle enzyme) was incubated in the standard assay system for 9 minutes with and without inhibitor. At this time ADP (1.25 µmol) was added, and the reaction was initiated at 10 minutes by addition of PEP (5 µmol). Each figure is the mean of the number of determinations given in parentheses. Each individual I_{50} value was obtained from not less than six inhibitor concentrations.

Inhibitor	Rabbit muscle	T.brucei TREU 277	T.rhodesiense EATRO 173	Concentration at which no inhibition is reported (mM)
Melarsen oxide	N.I. (4)	1.1×10^{-4} (7)	1.0×10^{-4} (4)	1
Phenylarsenoxide	N.I. (2)	2.5×10^{-4} (4)	2.6×10^{-4} (2)	1
p-aminophenylarsenoxide	N.I. (2)	11.2×10^{-4} (3)	13.0×10^{-4} (2)	5
Mel W.	N.I. (1)	3.4×10^{-4} (2)	3.1×10^{-4} (1)	1
Sodium melarsen	-	◇ (1)	-	-
Melamine	N.I. (1)	N.I. (2)	N.I. (2)	10
Sodium arsenite	N.I. (3)	N.I. (4)	N.I. (2)	100
N-ethyl-maleimide	-	3.8×10^{-4} (2)	3.1×10^{-4} (2)	-
pCMB	12.5×10^{-7} (3)	9.3×10^{-7} (4)	9.9×10^{-7} (2)	-
p-aminobenzoate	N.I. (3)	N.I. (2)	N.I. (1)	1
Iodoacetate	N.I. (1)	* (5)	N.I. (1)	1
Iodoacetamide	N.I. (1)	N.I. (2)	N.I. (1)	10

N.I. denotes not inhibited. : - denotes not determined. ◇ denotes 17% inhibition at 5 mM.

* denotes results variable, between 19% inhibition and 14% stimulation at 1 mM-iodoacetate.

material. Unpublished experiments of Fairlamb (1970) indicate that the level of ATPase activity is approximately 0.2 µmol /min/mg protein.

Obviously any investigation of the effects of these modifiers on the trypanosome PK would be severely hindered by the presence of these enzymes, and a programme for purification of the enzyme was prepared. It may, however, be noted that the consistency of the results obtained for the K_MADP in the crude system, is probably in part due to the high ATPase activity of the enzyme preparation, which would tend to maintain the concentration of exogenous ADP by continual removal of the ATP produced.

Effect of inhibitors

In view of the previously stated inaccuracies inherent in the use of the unpurified enzyme, the investigation of inhibitor action was confined to a brief survey. By comparison of these data (Table 19) with the inhibition characteristics of the purified enzyme (Table 24), it may be seen that the purification procedure does not alter the properties of the enzyme to any large extent.

A more detailed interpretation of the inhibition pattern portrayed by PK will be found following the analysis of the purified enzyme system, but certain points became apparent from the data shown in Table 19, in which the properties of the mammalian muscle PK are included for comparative purposes.

An obvious specificity for the arsenical drugs may be seen, in that the trypanosomal systems are preferentially inhibited, and the muscle enzyme is unaffected by these substances. On the other hand, p.chloromercuribenzoate (pCMB), a classical thiol inhibitor, affects all three systems to the same extent. Of interest also is the total lack of effect of arsenite, although here, as in the case of sodium melarsen,

correction had to be made for the presence of sodium ions which are inhibitory to the enzyme. The inability of melamine to inhibit the enzymes supports the hypothesis that it is the -As=O grouping of melarsen oxide which is the active part of the molecule. At this point it was thought that the preferential inhibition of the trypanosome enzyme was due to the involvement of a dithiol group in this protein, as opposed to a monothiol in the muscle enzyme, accounting for the inhibition of the latter by pCMB. Later data from the purified enzyme system cast some doubt on this interpretation.

Further discussion of these data at this point would be of little value, as most of this work was repeated on the purified material, and this table has been included solely for comparative purposes. However, as well as establishing a differential effect between host and parasite enzymes with regard to the arsenicals, it also shows that the monomorph- and pleomorph- derived enzymes are affected in an identical manner.

All six of the active inhibitors from this tabulation have I_{50} values which, within the range of experimental error, are equal for the two types of infection. The value of this identity lies in the following section of this thesis, on the purification of trypanosome PK. On the basis of these results, it was decided that lyophilised T.brucei TREU 277 would be used as the starting material for the purification process, as it is very much more rapid and simple to produce (relatively) large amounts of this strain, than it is to accumulate lyophilised T.rhodesiense EATRO 173.

Reaction with melarsen oxide

The I_{50} values quoted in Table 19 are subject to criticism on the grounds that the arsenicals will react with protein thiol groups other than those of PK, thus lowering the effective concentration of inhibitor

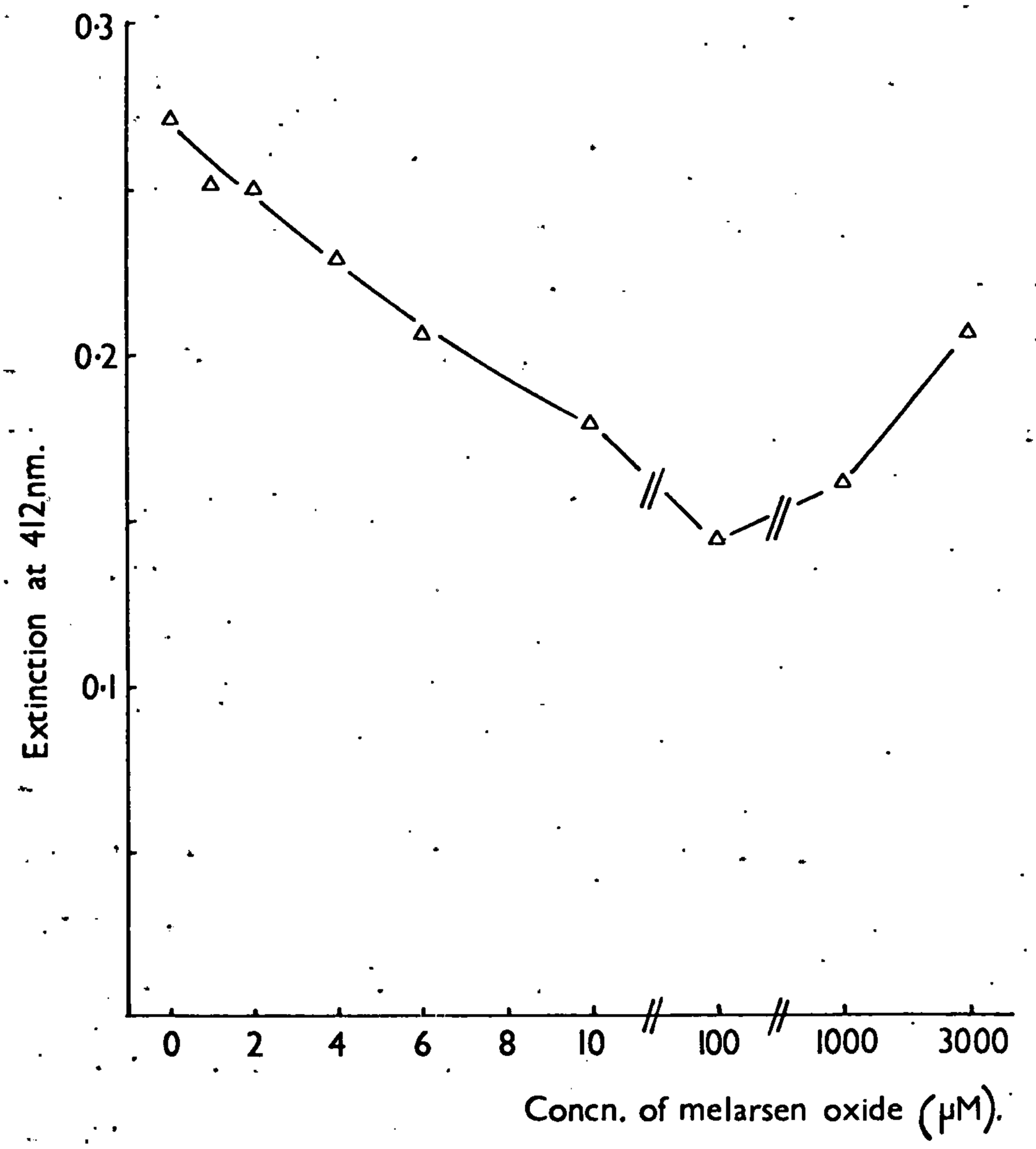


Fig. 14. Interaction of melarsen oxide with the thiol groups of lyophilised trypanosomes. Resuspended, lyophilised *T. rhodesiense* EATRO 173 was incubated in aqueous solution with melarsen oxide for 20 minutes prior to the estimation of free thiol groups by Ellmans reagent as described in Chapter 3.. The concentration of thiol groups is represented as the absorbance at 412nm of the final solution.

free in solution, and giving artificially high I_{50} values. One critical experiment which should have been carried out on the purified enzyme, was to show that on reaction with an arsenical, the free thiol content of the protein decreased. As will be discussed, technical difficulties have so far made this experiment impossible, and despite the objection mentioned in this paragraph, the interaction of melarsen oxide with the protein thiols was investigated using the crude enzyme material as an inferior alternative.

Figure 14 shows the decrease in free thiol present in the material after incubation with low concentrations of the arsenical for 20 minutes. The maximal coupling of the thiol groupings with melarsen oxide appears at approx. 10^{-4} M-inhibitor, at which concentration roughly 50% of the free thiol groups appear to be complexed. As the arsenicals were thought to be specific for dithiol groups, this figure appeared rather high, although reaction of a molecule of melarsen oxide with two monothiol-containing protein molecules is of course possible. The increase in free thiol concentration at higher inhibitor concentrations, while unexpected, may be accounted for by either of two hypotheses. At these high melarsen oxide concentrations, thiol groups which are not readily complexed with the arsenoxide group (e.g. monothiols) may react and thus cause a conformation change in the protein molecules thereby exposing previously "hidden" unreactive thiols. On the other hand, whereas at low concentrations of the inhibitor one arsenical molecule may be forming strong bonds with two thiol groups to produce a cyclic thioarsinite, in the higher range two arsenical molecules may be reacting with the same thiols to produce much weaker links between the arsenic atom and one sulphur atom. These bonds would be much more readily broken in competition with the Ellman's reagent and would thus increase the apparent concentration of free thiol.

Suffice to say, then, that reactions do occur between the trivalent arsenical trypanocide and thiol groups in the trypanosomal material, and between melarsen oxide and PK. The remainder of this work is devoted to the purification and characterisation of PK from T.brucei to obtain information regarding the molecular activity of the arsenical drugs.

4) PURIFICATION OF TRYPANOSOME PYRUVATE KINASE

The published methods for purification of PK are without exception based on materials which are readily obtained in large quantities (e.g. Tanaka et al., 1967a, 1½ - 5 kilos rat liver). This allows the utilisation of methods of purification giving good increases in specific activity but low total recovery of activity - in the paper cited above 6.1% M type and 1.6% L type PK were the final yields. In the case of trypanosomal material, the lyophilised starting material was available in milligram as opposed to kilogram quantities, and hence a different approach was necessary.

This lack of material probably accounts for the fact that at the commencement of this work, no report had been produced on the purification of an enzyme from T.rhodesiense or T.brucei. Seed and Baquero (1965), had centrifugally separated a T.rhodesiense lysate into sediment and supernatant to demonstrate the particulate nature of hexokinase, but no further purification was undertaken. Their preparation of organisms was also contaminated with blood elements.

To date, only one further purification of an enzyme from this organism has been described. Risby et al., (1969) obtained a 60-fold increase in the specific activity of phosphohexose isomerase (E.C. 5.3.1.9) by ammonium sulphate fractionation and DEAE-sephadex chromatography. In the context of the present work, it is noteworthy that

these authors state as their primary reason for choosing this enzyme for investigation, its demonstrable stability.

Many of the purification techniques which were tried and found to yield low recoveries of activity in this work, will only be dealt with in brief. To avoid the cataloguing of an excess of data, the quantitative results of these experiments will be confined to one set of conditions, and only the adopted procedure is described in detail.

Preliminary experiments

The use of 25% ($^{v}/v$) glycerol solutions for many trial experiments complicated manipulation of the material due to the high viscosity of the resuspension. Aqueous resuspensions of the freeze-dried material were therefore used despite the relatively low stability of the enzyme in this solution, as most preliminary experiments could be rapidly completed. An untreated aliquot of the lyophilised material resuspended in water, was routinely used as a control for the instability of the enzyme.

Solubilisation of pyruvate kinase.

The particulate nature of the resuspended freeze-dried material necessitated the inclusion of a solubilisation process as the primary step in the purification. At this stage, the desired combination of results from any treatment was a high recovery, with a high percentage of activity in the supernatant after centrifugation (3000g for 10 minutes). The method of solubilisation which best met these requirements was found to be Teflon homogenisation in the presence of deoxycholate as a solubilising agent. Triton-X 100, which gave slightly higher yields, interfered strongly with the estimation of protein spectrophotometrically at 260/280 nm and had to be replaced routinely with deoxycholate. When

TABLE 20

Non-polar solvent extraction of lyophilised T.brucei TREU 277

Lyophilised material was resuspended in water, 25% $^{v}/_{v}$ glycerol, or directly in the solvent, at 2°C and a concentration of 3 mg/ml. Aqueous and glycerol solutions were made 90% $^{v}/_{v}$ with solvent, and all solutions were homogenised. After standing for 10 minutes in ice with occasional agitation, the solutions were centrifuged for 10 minutes at 10,000g, and the sediments (in the cases of acetone mixtures and pure solvents) or aqueous layers removed. Sediments were air-dried and resuspended in 25% $^{v}/_{v}$ glycerol by homogenisation. Aqueous residues were made 25% $^{v}/_{v}$ with respect to glycerol and all samples were assayed in the standard system.

Specific activity of starting material = 0.78 µmol/min/mg protein.

	Non-polar solvent					
	Acetone		Benzene		Diethyl ether	
Primary solvent	Yield (%)	Sp.act^{y} (µm/min/mg prot.)	Yield (%)	Sp.act^{y} (µm/min/mg prot.)	Yield (%)	Sp.act^{y} (µm/min/mg prot.)
Water	28	0.24	36	0.32	37	0.32
25% ($^{v}/_{v}$) glycerol	54	0.51	42	0.40	51	0.49
Non-polar solvent	78	0.62	83	0.66	92	0.72

this procedure was carried out in TGD, the supernatant yield was from 87% - 96% of the activity. This supernatant, however, was exceptionally glutinous and was totally unsuitable for further purifications involving column separation of protein fractions. A primary treatment of the lyophilised material was therefore necessary, to decrease the viscidity of the preparation which is probably due to the presence of large amounts of flagellar protein and the coacervation of protein by cellular lipid.

Treatment with non-polar solvents.

Application of Teflon/deoxycholate homogenised samples to columns of DEAE-cellulose, Sephadex or Biogel, repeatedly resulted in obstruction of the columns, and elution of the small amount of activity which was recoverable, in one peak at the solvent front. It was found that a suitable pretreatment of the material to remove this difficulty was prior extraction with a non-polar solvent. The results of extractions with acetone, diethyl ether and benzene may be seen in Table 20.

From inspection of these results, it appears that solvent extraction in aqueous solution is unproductive, and direct ether extraction of lyophilised material was incorporated as the first step in all subsequent procedures. The yield of activity at this step varied from 89% to 98% and was maintained in the higher part of this range by previous drying of the ether over anhydrous sodium sulphate. The sedimentation of protein material after ether extraction was found to be complete after five minutes centrifugation at 3000g, and these conditions were routinely used for convenience.

Ethanol fractionation.

As may have been expected from the low recoveries of the trypanosome PK from acetone- or ether-water mixtures (Table 20), the loss of

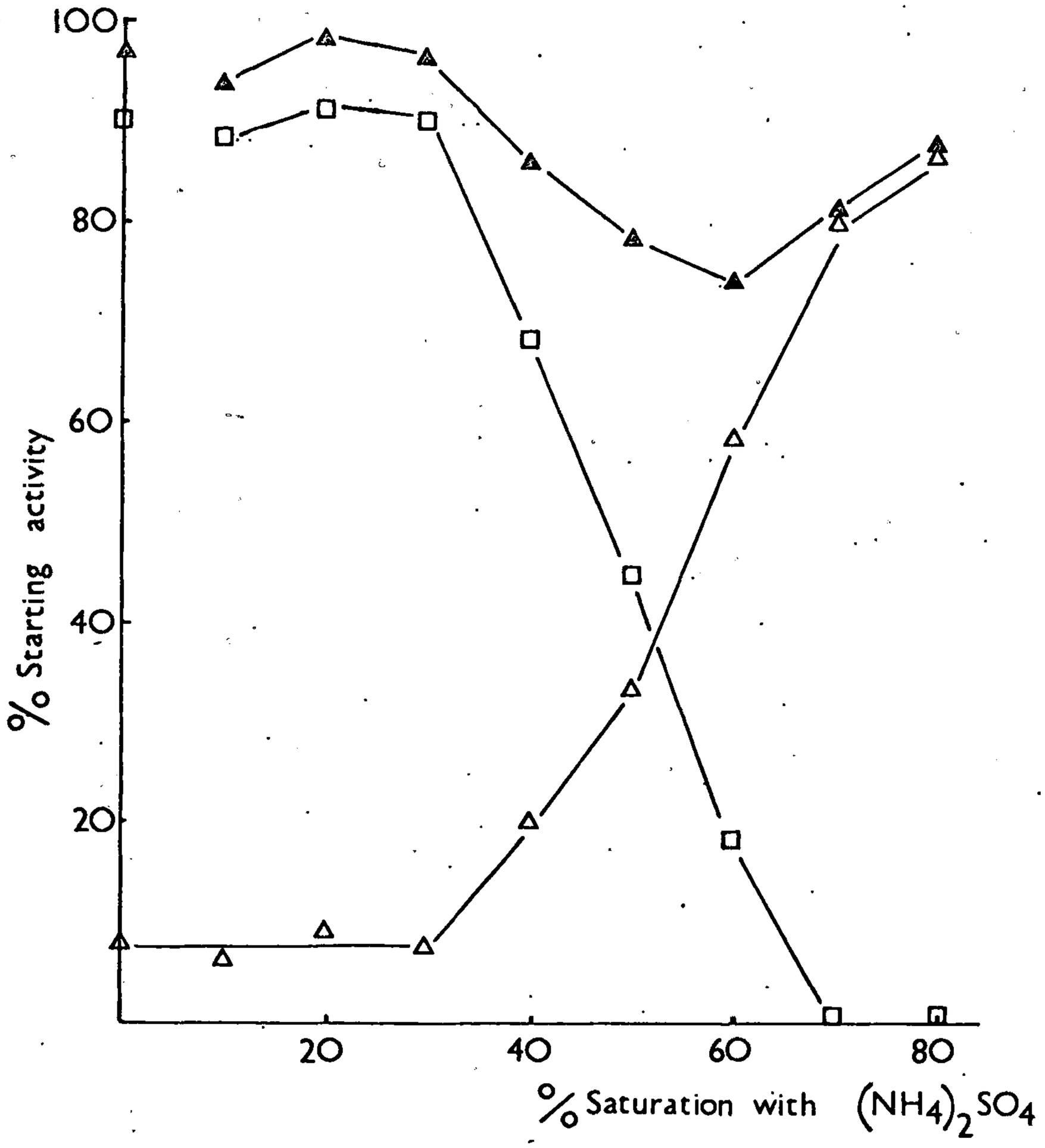


Fig. 15. Ammonium sulphate fractionation of lyophilised T. rhodesiense EATRO 173. Lyophilised material was ether-extracted and resuspended in water at 2.0 mg/ml. Finely ground solid ammonium sulphate was added to aliquots of the pre-cooled suspension in ice, to concentrations up to 80% saturation. After standing for 20 minutes in ice, with occasional stirring, the incubations were sedimented for 10 minutes at 1000g and the supernatants removed for assay. Sediments were resuspended in a volume of water equal to that of the original solution. All assays were carried out in the standard assay medium with 1.25 µmol ADP and 5.00 µmol PEP, and were initiated by addition of enzyme. Rates are plotted as % starting activity. Δ = sediments. □ = supernatants. ▲ = total activity recovered.

activity accompanying dilution of the resuspended material with ethanol was too great for the method to be of use in this work. A differential precipitation of protein could be obtained between 25% ($^{v}/v$) and 50% ($^{v}/v$) ethanol at -10°C, using a 25% ($^{v}/v$) glycerol resuspension of ether extracted material, but no activity was recovered in the precipitated protein.

Fractional denaturation by heat.

Purification of rabbit muscle PK by Tietz and Ochoa (1962) involved denaturation and insolubilisation of contaminating proteins by heating of the material at 60 - 65°C. After one minutes incubation at this temperature, $\leqslant$ 16% of the activity was recoverable from aqueous or glycerol solutions of the trypanosome enzyme. Heating at lower temperatures similarly destroyed the enzyme without removing significant amounts of protein from solution.

Neutral salt fractionation.

The response of trypanosomal PK to ammonium sulphate fractionation is described in Figure 15. The minimum in the total recovery curve is still of doubtful cause. Ammonium ions are known to be able to replace potassium ions as activators of PK, but as the volume of enzyme used per assay was 0.10 ml, the final concentration of ammonium ion never exceeded 3% saturation (0.16 M). Standard enzyme assays, run with 3% ammonium sulphate present in the assay cuvette, showed no significant effect at this salt concentration.

Later experiments on ammonium sulphate fractionation using a 40 - 60% or a 40 - 70% cut, gave higher yields of activity in the precipitated fractions, varying from 80 - 90% of the total activity in a 40 - 70% fraction, and from 60 - 80% in the 40 - 60% sediment.

The primary objective of these purification procedures, namely high yields of activity, was therefore met to a satisfactory degree by this technique. The activity of the above sediments was therefore correlated to the protein content, and the results of two representative experiments are tabulated below.

Protein was estimated by the standard procedure on the original material, and on the fractions, after the latter were dialysed for one hour against water (2 changes) to remove interference from ammonium sulphate.

Fraction	Units activity	Protein (mg)	Yield	Specific activity (units/mg protein)
Total resuspension*	9.32	16.08	-	0.58
40 - 60%	6.41	3.75	69%	1.72
40 - 70%	7.65	8.14	82%	0.94
Total resuspension*	9.35	11.42	-	0.82
40 - 60%	6.90	2.56	74%	2.74
40 - 70%	8.22	5.10	88%	1.61

* Activity measured at the same time as the dialysed fractions.

When a similar fractionation was attempted on a 25% (v/v) glycerol solution of the enzyme, very different results were obtained. The solubility of ammonium sulphate is greatly diminished by the presence of glycerol, and at salt concentrations approaching saturation in this solvent, the majority of enzyme activity remained in the supernatant and practically no protein precipitate was obtained. When higher centrifugal forces (up to 10,000g for 10 minutes) were employed to separate the fractions, enzymic activity was sedimented in parallel with the protein, giving no significant increase in specific activity.

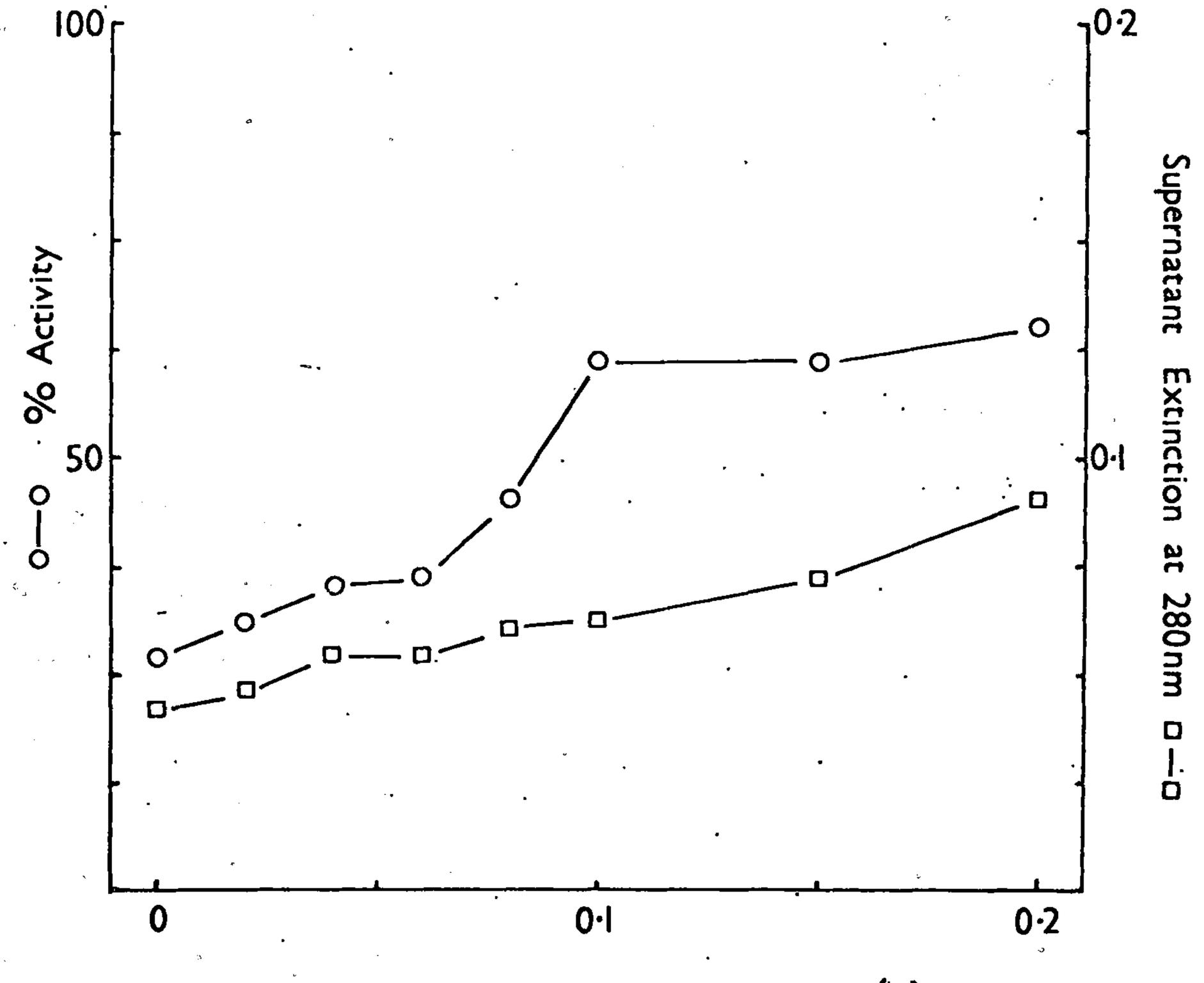


Fig. 16. The elution of pyruvate kinase from DEAE-cellulose. Ether extracted, lyophilised T. rhodesiense EATRO 173 was resuspended in 25% (v/v) glycerol at 2.5 mg/ml. To aliquots of this material was added DEAE-cellulose (1gm wet wt./5.0 ml soln.) pre-swollen in the same solvent, and the mixture was allowed to equilibrate for 20 minutes in ice after adjustment of the pH to 7.3 with 0.1M-KOH/HCl. KCl (1.0M) was added to give the final concentrations indicated after equalisation of the volumes with 25% (v/v) glycerol. After a further 20 minutes the samples were centrifuged briefly at 200g to remove the DEAE-cellulose and the supernatants were assayed for activity in the standard assay system. The protein content of the supernatants is represented as the extinction at 280nm.

Aqueous ammonium sulphate fractionation therefore appeared to be a possible method of purification, under controlled conditions. However, experiment showed that the extraneous protein removed by this technique was eliminated on a Biogel column at a later stage of the purification, and to avoid unnecessary losses of activity this step was removed from the purification procedure.

Column chromatography

i. / DEAE-cellulose

The successful purification of PK by use of DEAE-cellulose column chromatography, has been reported from various enzyme sources (e.g. Washio & Mano, 1960; yeast). Despite the use of ether extraction as the primary step in the purification procedure, permanent adhesion of the enzyme to a column of DEAE-cellulose still occurred, unless a previous partial purification on CM-cellulose or Biogel was carried out. Some indication of the elution properties of the enzyme by a salt gradient was obtained from the use of a batch addition technique, the results of which are plotted in Figure 16. The release of activity is very much less sharp using this technique, than that obtained by use of a column procedure after CM-cellulose and Biogel-A treatment. Attempts were made to improve this process by alteration of equilibration time, the enzyme/cellulose ratio and by elution by changes in pH. Neither the yield of activity nor the sharpness of the elution profile was successfully increased.

ii. / CM-cellulose.

The blockage of DEAE-cellulose columns by the resuspended ether-extracted material was not thought to be entirely due to the viscosity of the applied samples. It appeared that an interaction between the

protein solution and the column material was causing a denaturation and precipitation of the protein, thereby producing the gelatinous "plug" at the top of the columns. It was thought possible that this non-specific precipitation was inhibiting the release of the absorbed PK, and CM-cellulose columns were therefore used to allow this precipitation to occur under conditions where the kinase itself will not be absorbed to the column.

Application of the resuspended ether-extracted material to a CM-cellulose column, and elution with TGD gave yields of activity in the range of 80 - 90% and a purification factor of approximately three-fold. The active eluate from such a column was found to be diluted by a factor of 4 to 8 fold, and required concentration before further use. (see Figure 17)

To concentrate this material, unswollen Sephadex G-10 was added to the solution and allowed to swell, before centrifugation and removal of the gel. However large losses of activity occurred on contact with the Sephadex, and this technique was abandoned in favour of the use of polyethylene glycol ("Carbowax"), as described under the final purification procedure.

This concentrated eluate was found to be ideal for application to Sephadex, Biogel or DEAE-cellulose columns to effect further purification. Dilution of the application to the CM-cellulose column by a factor of five- or ten-fold, to simulate the eluate dilution, did not improve the activity yields and slightly decreased the purification factor. Simple dilution of the resuspended ether-extracted material and subsequent re-concentration did not yield any diminution of the gelatinous properties of the material, so the removal of extraneous protein on the column appears to be the critical factor.

The ion-exchange properties of the CM-cellulose column appear to be involved in a normal fashion in the absorption of unwanted protein, as replacement of the column by one of finely powdered cellulose did not give a useful separation or purification. However, the shape of the elution profile from the CM-cellulose column (Fig.17) leads the author to believe that such a column is acting, at least partly, as a filter, whose ion-exchange properties are secondary but necessary.

iii. / Gel filtration

Many variations of conditions were employed in attempts to effect a purification using Sephadex G-150, Sephadex G-200, and the spherical agarose gel, Biogel-A, in various mesh sizes. On samples not previously treated with CM-cellulose, yields were consistently very low (< 20%), and elution profiles were with one exception confined to a single protein peak. The exception was the use of Biogel A-150 with the 40% - 60% cut from an ammonium sulphate fractionation as the column application. This produced three peaks on the elution pattern, but the activity yield was only 18%.

After treatment with a CM-cellulose column, however, a very satisfactory separation was obtained with the agarose columns, with good yields of activity. Sephadex G-200 gave rather poorer separations and lower yields with the result that this method was discarded.

No benefit would accrue from further extended description at this point of variations in elution procedure and profiles obtained with methods now abandoned, and a description of the full purification procedure adopted follows.

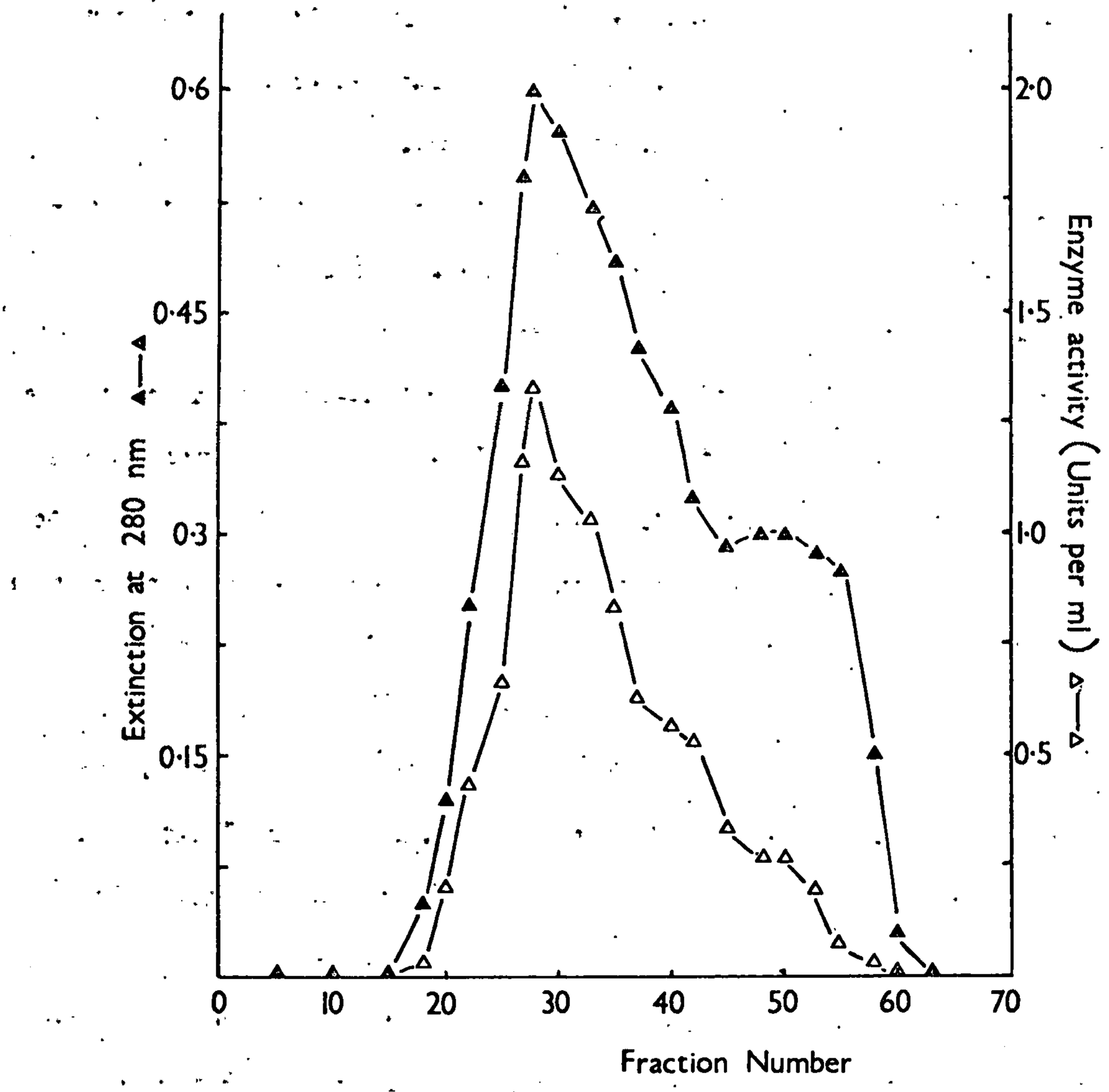


Fig. 17. The elution of pyruvate kinase from CM-cellulose. The column of CM-cellulose was pre-eqilibrated and eluted with TGD. Pretreatment of the material and chromatographic details are described in the adjacent text. Enzyme activity was measured in the standard assay system.

Purification procedure adopted for trypanosome pyruvate kinase.

On the basis of the trial procedures described in the previous section, and many others, the following routine was chosen as a standard method for purification of the enzyme.

Lyophilised material (100 - 150 mg) was resuspended in peroxide-free, sodium sulphate dried, precooled ether, at a concentration of 1 mg/ml. After homogenisation in a cooled glass mortar with a Teflon pestle, the material was allowed to stand for ten minutes in ice before rehomogenisation, and centrifugation at 3000g for five minutes. The ether layer was removed by suction and the extracted material dried in a current of air. The dried residue was resuspended at a concentration of 8 - 10 mg/ml in TGD and applied to a column of CM-cellulose, of length 30 cms and total volume approximately 240 ml. Elution was carried out with TGD, the column being pumped at a rate of 40 ml/hour, and 100-drop fractions were collected on an LKB "Ultrorac" fraction collector fitted with a "Uvicord" ultraviolet 260 nm scanner. As the material in this state contained a large amount of nucleic acid, the fractions were reassayed manually on a Unicam SP500 or SP800 spectrophotometer to obtain their absorbances at 280 nm, and so detect more accurately the fractions containing protein. As TGD interferes with the absorbance at 280 nm due to the presence of unknown quantities of oxidised DTT, fraction 1 or fraction 80 from the column was used as a blank, zero value. Aliquots of selected fractions were assayed for enzymic activity in the standard assay system, and a typical elution profile is shown in Figure 17. The abscissa is plotted in terms of the fraction number as opposed to elution volume, due to the variability found between individual fraction volumes as a result of variation in surface tension during elution.

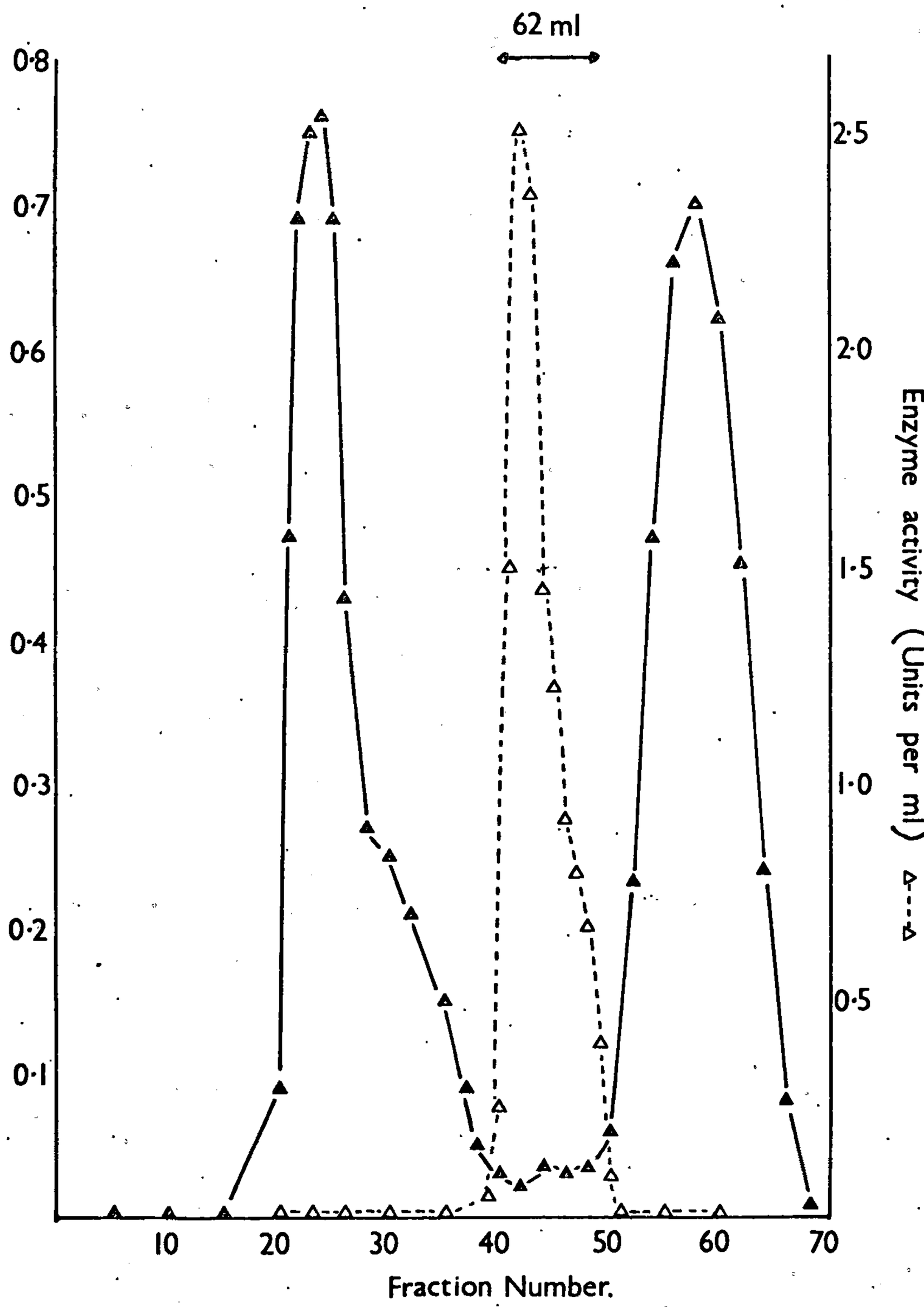


Fig. 18. The elution of pyruvate kinase from Biogel-A (150-200 mesh). The column of Biogel-A was pre-equilibrated and eluted with TGD. Pretreatment of the material and chromatographic details are described in the adjacent text. Enzyme activity was measured in the standard assay system.

The active fractions from the CM-cellulose column were combined. Although a greater purification is obtained at this stage if the second protein "peak" is omitted, it was found that increasing the specific activity in this manner at the expense of the yield of enzyme, did not significantly alter the purification obtained overall; the extraneous protein in fractions 45-55 of the eluate, is removed at the next stage of the purification.

No amount of care in the preparation, loading or elution of the column decreased the spreading and tailing of the protein peak, and it is suspected that precipitation and resolubilisation are occurring on the column. Elution of the column, after removal of the primary peak, with a KCl gradient from zero - 1 M-KCl in TGD, produced varying amounts of protein, in up to three further peaks, none of which were enzymically active. The position and number of these peaks were irreproducible, and they are not included in Figure 17 as a result. The total amount of protein eluted in this fashion was never in excess of 25% of that applied, and was in most cases between 10 - 15%.

The combined active fractions were placed in a preboiled dialysis sac, and immersed in "Carbowax" for 12 - 24 hours in a cold room, the time in any individual case being that required to reduce the volume to 20 - 30 ml. After assay, this material was applied to a column of Biogel A, (150 - 200 mesh, 50 cm in length, approximately 400 ml in volume), which had been previously equilibrated in TGD. Elution from such a column with TGD gives a profile such as that shown in Figure 18, which was obtained at a controlled flow rate of 40 ml/hour. In some fractionations, a small amount of activity ($\leqslant$ 10%) appeared as a peak coincident with the first protein peak shown on this diagram. On one such sample, the enzyme appearing at this point was collected, subjected

TABLE 21

Purification of trypanosome pyruvate kinase

Details as described in the text. Protein estimated by the method of Lowry et al., (1951) (fractions 1-4) or by the method of Warburg and Christian (1942) (fractions 5 & 6). Fractions 3 & 4 were dialysed for four hours at 2°C against two changes of water prior to estimation of protein. Activity was measured in the standard assay system.

Treatment	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg protein)	Yield Step	Yield Total (%)	Total Purification
Starting material	-	148	119	0.80	-	100%	-
Ether extract	20	144	111	0.77	93	93	zero
CM-cellulose eluate	163	52	100	1.92	90	84	2.5
CM-cellulose fractions after "carbowax"	27	50	94	1.88	94	79	2.5
Biogel-A fractions	62	~1	75	75	80	63	~90
Biogel-A fractions after "carbowax"	23	~1	70	70	93	59	~90

to "Carbowax" treatment, and shown to have the same basic kinetic properties as the major enzymic fraction. Although the properties of this minor and variable fraction were not investigated in such detail as those of the major activity peak, sufficient evidence was obtained to suggest that this minor peak is due to binding of the PK to some other protein element in solution, rather than to the existence of an isoenzymic form of the enzyme. This enzyme had the same K_M ADP, S_{50} PEP and FDP-activation characteristics as the enzyme from the major peak.

The combined active fractions of the major activity peak were routinely subjected to treatment with "Carbowax" as described for the CM-cellulose fractions, as the enzyme was found to be more stable in the concentrated form. The final purified material was optically clear, and unfortunately did not contain adequate amounts of protein for estimation purposes. The interference of DTT and its oxidation products with the spectrophotometric estimation of protein at 280 nm made this method unreliable. However, an approximation to the protein content could be obtained by the method previously described for the CM-cellulose fractions. The purification data for a representative run, (shown in Figures 17 and 18) are given in Table 21.

Comments on the purification scheme.

i. The graphical representation of the Biogel-A elution profile shown in Figure 18 is of one of the later purification processes. Previous attempts, in which the elution rate of the column and the ionic strength of the elution medium were varied, gave quantities of protein in the enzyme peak varying from about 2 - 5 mg protein, as estimated from the ratio of the absorbance at 280 nm of this fraction to the overall

absorbance at 280 nm of the application. For the kinetic studies reported below, a satisfactory minumum purification factor of sixty-fold was set as the final requirement.

Variation of the conditions employed in running the Biogel-A column did not increase the enzymic yield, and as mentioned above, occasionally altered the protein elution profile. The inclusion in the TGD system of 0.007 M-$MgSO_4$ and 0.07 M-KCl, for example, shifted approximately 60% of the 280 nm-absorbing material from the first to the second major protein peak, and simultaneously produced a third minor peak in the region of the pyruvate kinase active fractions. This information, similar to that obtained on ammonium sulphate fractionation of the lyophilised material, may be of use in the preparation and purification of other trypanosomal enzymes.

ii. Application of the active fraction from a Biogel A column to a column of DEAE-cellulose was found in later purifications such as that described here, to be unnecessary. In earlier purifications, where the active peak contained measurable amounts of protein, further purification of the enzyme on DEAE-cellulose was successfully carried out, the enzyme eluting at approximately 0.1 M on a KCl gradient. Yields from such a procedure were low ($< 50\%$) and this step was avoided whenever the purification on the Biogel A column was adequate.

iii. The final preparations from Biogel A, from three batches of lyophilised material, were found to be free from the following enzymes: hexokinase, enolase, fructose 1;6 diphosphatase, aldolase, ATPase and any other enzymes utilising PEP. Carbon balances carried out with the purified material gave the following results, the method being identical to that described previously in this chapter.

Batch	µmoles PEP utilised	µmoles pyruvate formed	% yield
1	1.07	1.05	99%
2	1.36	1.37	100%

Kinetic studies involving variation of PEP concentration using the purified material appear therefore to be valid.

Preparation of the purified enzyme for kinetic investigation.

The final enzyme preparation, produced by the procedure developed above, is in a buffered 25% ($^{v}/v$) glycerol solution containing 10^{-3} M-DTT and to produce a conveniently measurable rate of approximately 0.1 µmole per minute, up to 50 µl of this freshly prepared solution is required per assay. In an assay volume of 3.0 ml, the final concentration of DTT will therefore be of the order of 2×10^{-5} M. As concentrations of the arsenicals in the region of $10^{-5} - 10^{-4}$ M were necessary to produce 50% inhibition, removal of the stabilising thiol reagent prior to assay of the enzyme was essential, to avoid complexing significant quantities of the arsenical with this reagent in competition with the enzyme thiol groups.

The enzyme was found to be very much more unstable in the purified form, especially in the absence of DTT, than in the crude material under similar conditions. However, the following procedure was developed for removal of the thiol.

A column of Sephadex G-25 was prepared, the gel being swollen in 0.1 M tris pH 7.2/25% ($^{v}/v$) glycerol and thoroughly degassed by boiling under reduced pressure prior to preparation of the column. The degassing procedure was carried out under a layer of light liquid paraffin (ρ = 0.83 - 0.87) and the column (18 cm long; volume 50 ml) was prepared under the same material. An aliquot of the concentrated

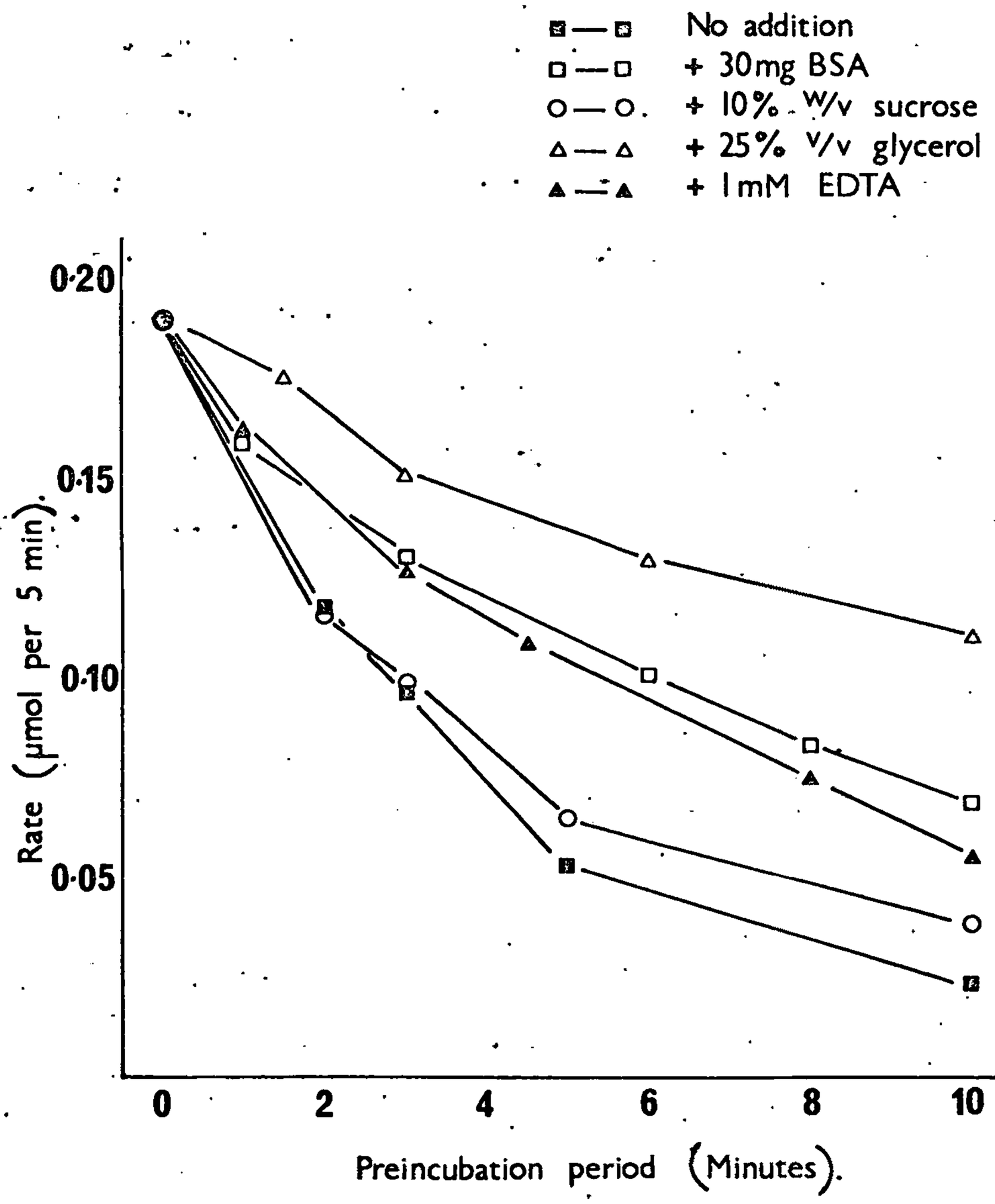


Fig. 19. The stabilisation of purified pyruvate kinase in the assay medium. Aliquots of enzyme (50 µl) were added to the standard assay medium in the presence of the stabilising agents as indicated. After preincubation the reactions were initiated by addition of ADP (1.25 µmol) and PEP (5.00 µmol), except in the case of the zero-time figure which was obtained by starting the reactions by addition of enzyme.

Biogel-A eluate (2 - 3 ml) was applied to this column, eluted with deoxygenated Tris/glycerol and fractions were collected under paraffin. Under these conditions, the enzymic yield was from 40 - 60%, and the enzyme in the final solution had a half-life of approximately four hours compared to the half-life of about 3 days in the concentrated Biogel-A eluate containing DTT. Where a column of this type was used more than once, thorough washing with a solution of sodium dithionite after use, followed by re-equilibration in degassed Tris/glycerol markedly increased the yield of activity on the second and subsequent runs.

Removal of DTT did not affect the kinetic properties of PK, and this procedure was therefore only followed for investigation of the action of inhibitors on the enzyme.

5) KINETIC PROPERTIES OF PURIFIED TRYPANOSOME PYRUVATE KINASE.

Conditions of assay

Early results obtained with the purified preparation were to some extent irreproducible, and the kinetic data obtained were subject to large errors. This was found to be due to the fact that despite the relative stability of the enzyme on storage in TGD, the enzyme was exceptionally unstable on dilution in the assay medium. This instability could be diminished by a number of substances, most of which interfered with the inhibition of the enzyme by melarsen oxide, and were therefore of little use. The effects of four stabilising agents are shown in Figure 19.

The activity of the unprotected enzyme is almost totally lost, in this case, after approximately 7 - 8 minutes preincubation in the absence of PEP. This time interval was found to be variable between preparations

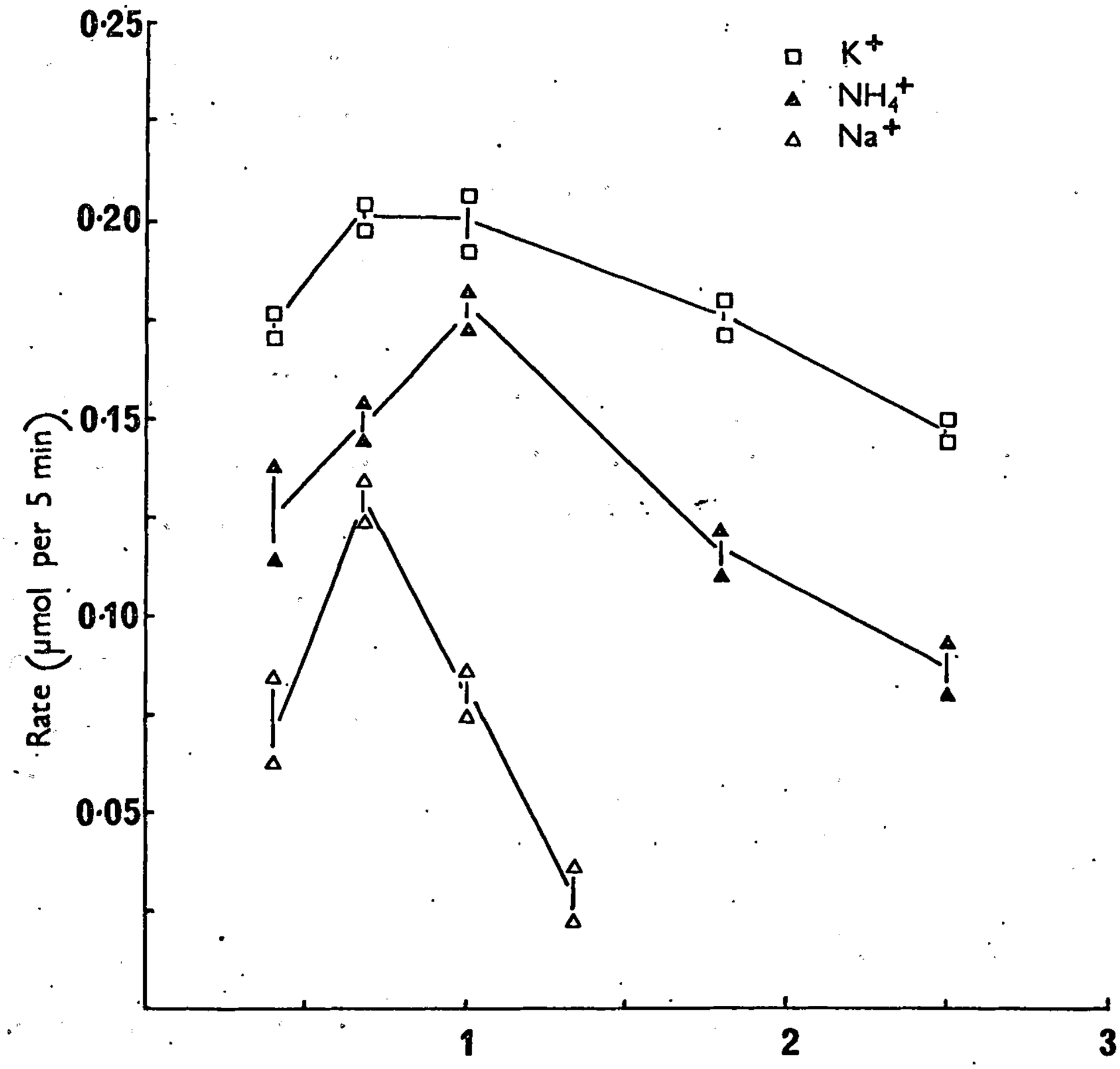


Fig. 20. The effect of monovalent cation concentration on the activity of purified pyruvate kinase. Aliquots of enzyme (100 μl) were assayed at the indicated cation concentrations in the presence of 6.67mM-$MgSO_4$. All other assay conditions were standard and reactions were initiated by addition of enzyme. Monovalent cations were added as the chloride salts.

of purified enzyme, and extended from 12-15 minutes with fresh material to 4-6 minutes after storage for 3 days.

Glycerol, PEP, BSA and EDTA all significantly stabilised the enzyme in the assay medium, but EDTA alone had the property of stabilising without protecting against the inhibition by melarsen oxide. The other three reagents all showed a protective effect against the arsenical. Following these results, EDTA (final concentration 10^{-3} M) was included in all assay media.

After storage of the enzyme in TGD for 3 days, the cooperative kinetic effects of PEP and FDP decreased markedly. Up to this time, the properties of the enzyme with respect to these effectors appeared to be stable, although the sensitivity to melarsen oxide decreased over the whole period of storage. Wherever possible, the enzyme preparations were therefore used within 48 hours of purification, and samples were routinely discarded after 72 hours.

Effect of monovalent cations.

Prior to investigation of the cation requirements of the enzyme, it was necessary to remove the cation contaminants from the enzyme solution, again by treatment on a Sephadex G-25 column. Elution from the column in this case was carried out with 25% (v/v) glycerol/.05 M-tris pH 7.2 and the substrate, coenzyme and buffer in each case were neutralised with the appropriate base. The LDH solution used was dialysed for six hours against three changes of distilled water to remove ammonium ions, the commercial preparation being stabilised in 2.2 M-ammonium sulphate.

Despite these precautions, the apparent residual activity in the absence of exogenous monovalent cation probably indicates some contamination with potassium ions rather than the ability of the enzyme to

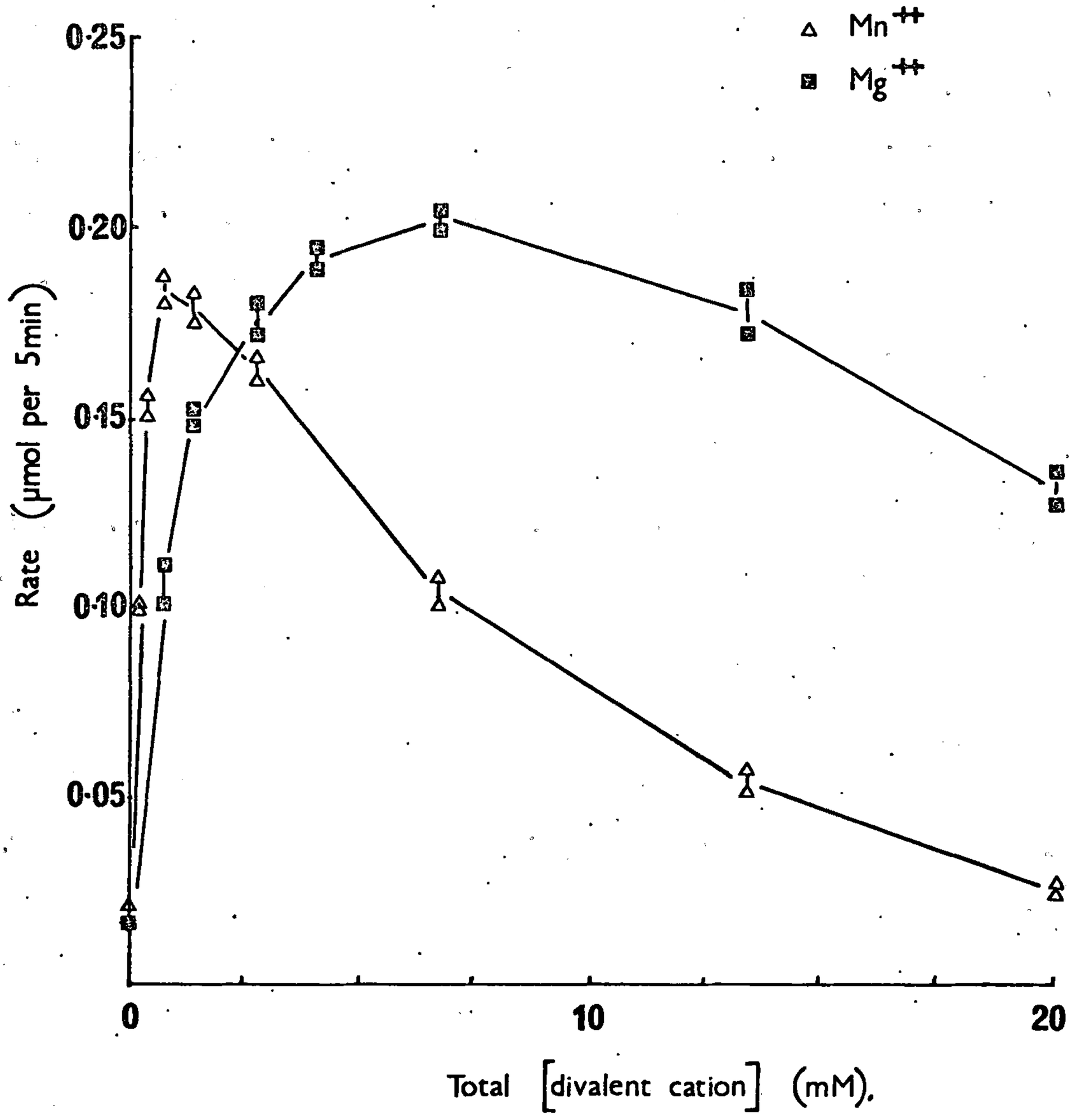


Fig. 21. The effect of divalent cation concentration on the activity of purified pyruvate kinase. Aliquots of enzyme (100 μl) were assayed at the indicated cation concentrations in the presence of 66.7mM-KCl. All other assay conditions were standard and reactions were initiated by addition of enzyme. All reagents were titrated to the assay pH with 0.1-M KOH. Divalent cations were added as the sulphate salts.

function in the complete absence of the ions (Figure 20). To prove a strict requirement would require a more stringent use of deionised water, and further use of an inactive base for reagent titration (c.f. Kayne & Suelter, 1965; $(CH_3)_4 N^+ Cl^-$). As, however, the major function of these tests was to determine optimal conditions for assay, 67 mM-KCl as in the standard assay medium was used routinely, and no further investigation of the monovalent cation requirement was carried out.

Effect of divalent cations

The PK of muscle and erythrocytes has been shown to be partially activated by Mn^{2+} ions as a replacement for the more commonly used Mg^{2+} salts (Boyer, 1962; Solvonuk & Collier, 1955). Figure 21 shows the relationship between added cation (in the form of the sulphate salt) and reaction rate, in the presence of 67 mM-KCl. On the basis of these figures, 6.7 mM-Mg^{2+} was retained as the optimal divalent cation concentration.

From data of this nature, it should be possible to determine the predilection of the enzyme for free Mg^{2+}, free ADP^{3-}, and the $MgADP^-$ complex, but two factors make this analysis impracticable. Firstly, in the absence of exogenous divalent cation, the activity of the preparation does not decrease to the expected zero rate. Although extrapolation of the data enables an approximation to be made of the levels of contaminating cations, the errors involved contribute greatly to the inaccuracy of the estimation of final $MgADP^-$ complex concentrations at low cation concentrations. Secondly, the significant part of these data for purposes of establishing the concentration of free ADP^{3-} lies at these very low levels of Mg^{2+} ion, and the error in estimation of these slow enzymic rates contributes an extremely large measure of inaccuracy to the extrapolation

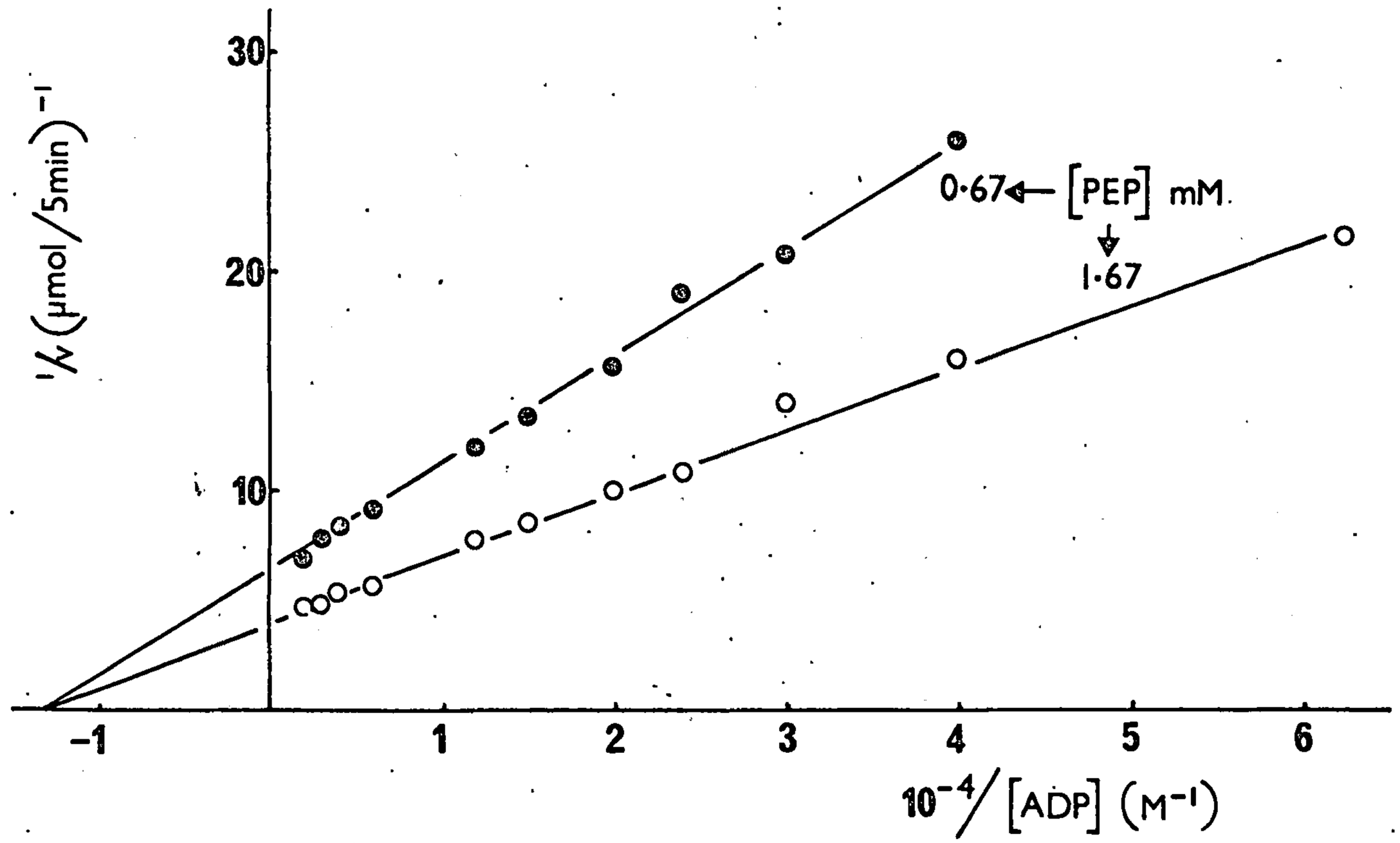


Fig. 22. The effect of ADP concentration on the activity of purified pyruvate kinase. Aliquots of purified enzyme were preincubated in the standard assay medium for 3 minutes at 25°C. Reactions were initiated by addition of ADP and PEP as indicated. Initial velocities were measured and Lineweaver-Burk plots derived as described in Chapter 3. 0.1 ml of enzyme was used per assay.

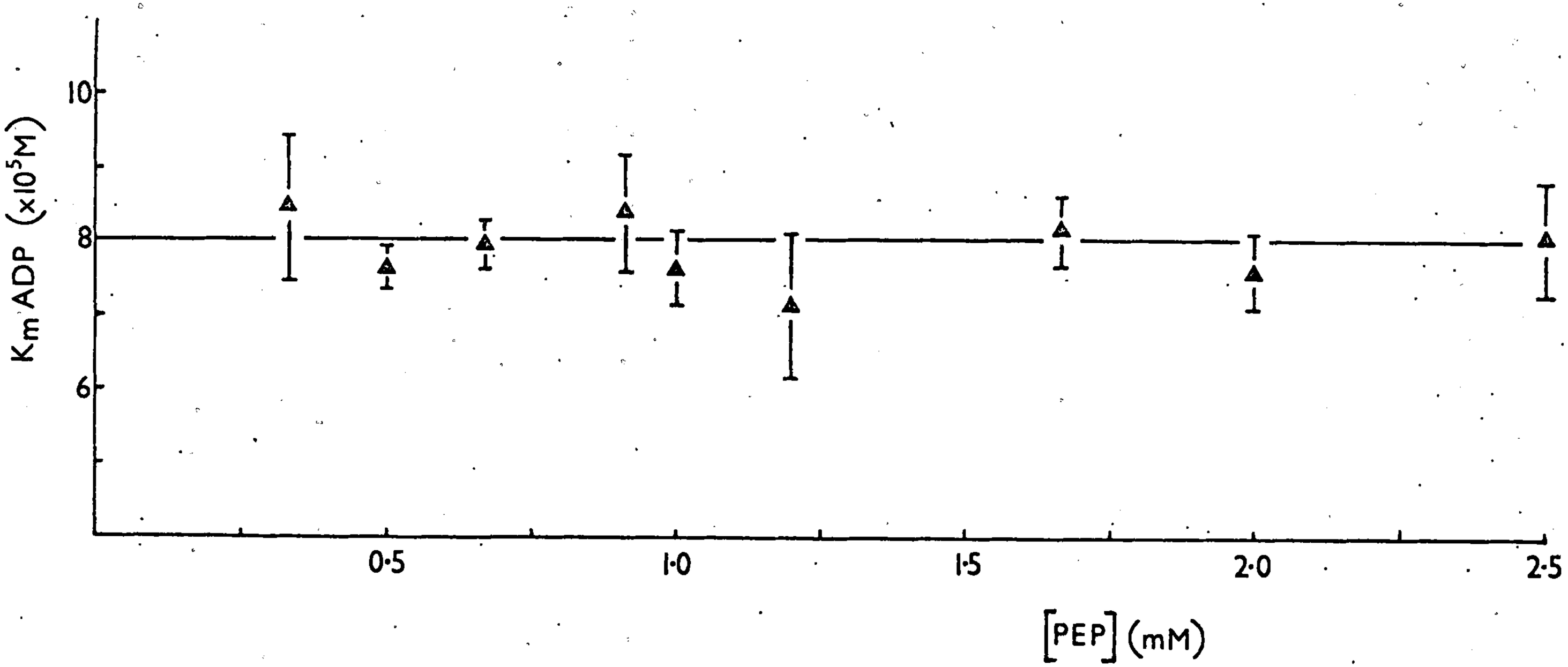


Fig. 23. The effect of PEP concentration on the K_mADP value of purified pyruvate kinase. The data were obtained as for Fig. 22. Each value is from a regression on not less than seven experimental points, and is plotted ± the standard deviation.

mentioned above. At the Mg^{2+} concentration used routinely for enzyme assay, (6.7 mM) all the ADP present is in the form of $MgADP^-$.

Both cations inhibit the kinase in high concentration, although the tolerable level of free Mg^{2+} exceeds that of free Mn^{2+}. Apparent half-saturation values are approximately 6.7×10^{-4} M-Mg^{2+} and approximately 1×10^{-4} M-Mn^{2+}.

Effect of nucleotide coenzymes.

Two double reciprocal plots describing the effects of ADP concentration on the enzyme activity are shown in Figure 22. The weighted regression lines fitted to these data give extrapolated K_M ADP values of $8.3 \pm 0.4 \times 10^{-5}$ M-ADP (1.67 mM-PEP) and $8.0 \pm 0.3 \times 10^{-5}$ M-ADP (0.67 mM-PEP). The mean value, obtained in the presence of 1.67 mM-PEP, from seven purified batches of enzyme, was $K_M = 8.2 \times 10^{-5}$ M-ADP with a standard error of the mean of 0.5×10^{-5} M. These figures, as discussed in the section pertaining to the crude enzyme system, are in reality the K_M values for the $MgADP^-$ complex, and they appear slightly lower than those obtained with the unpurified system.

The independence of the K_M value for the coenzyme of the concentration of substrate over the range 0.33 - 2.5 mM-PEP is shown in Fig. 23. This basic pattern for a bireactant system indicates a sequential mechanism in which both substrates are bound to the enzyme prior to release of a product, no irreversible step intervening between the combination of the first and second cosubstrates. Further discussion on the mechanism of this enzyme will be found in Chapter 6, the only other point to be made here being that no inhibition was found by ADP up to a final assay concentration of 4.5×10^{-4} M.

TABLE 22

Nucleotide specificity of purified trypanosome pyruvate kinase

Apparent K_M and V_{max} values were determined as in Figure 22, the data being treated as described in Chapter 3. K_M app. values are quoted as the mean of six determinations on a total of four enzyme batches, with the exception of ADP which is from seven determinations on seven enzyme batches. V_{max} values are from one representative sample, on which three estimations were carried out. Both parameters are mean values $\pm$ SEM. All assays were carried out at 1.67 mM-PEP.

Nucleotide	K_M (app) (mM)	V_{max} (µmol/min/ml enzyme)
ADP	0.082 $\pm$ 0.005	1.46 $\pm$ 0.11
GDP	0.098 $\pm$ 0.010	1.40 $\pm$ 0.16
UDP	0.410 $\pm$ 0.130	1.39 $\pm$ 0.42
CDP	2.53 $\pm$ 1.12	1.43 $\pm$ 0.62

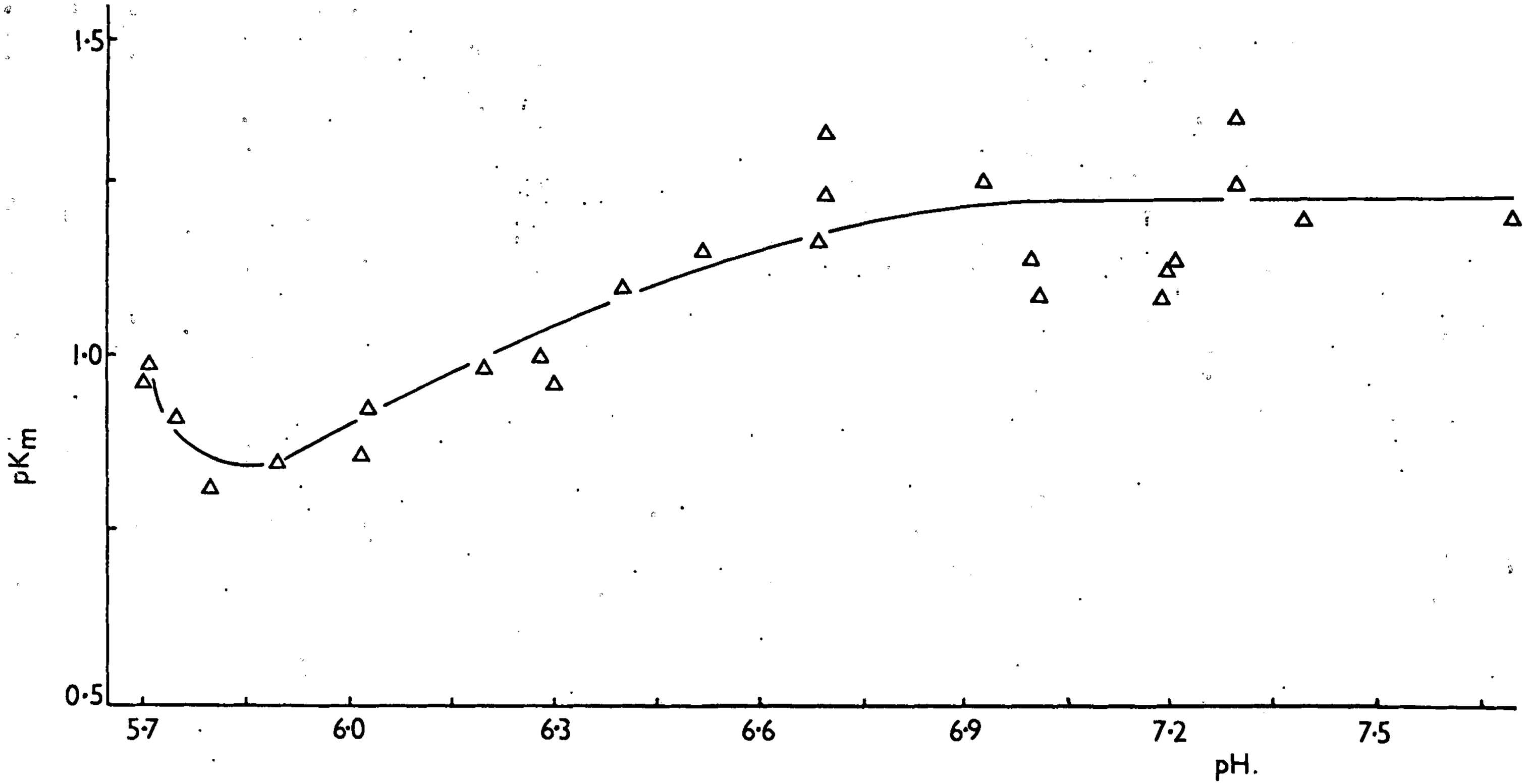


Fig. 24. The effect of pH on the K_mADP value of purified pyruvate kinase. Sufficient volumes of all assay constituents except ADP for ten assays were mixed, titrated to the required pH with 1.0M-KOH/HCl and the mixture divided into the correct volumes for nine assays. ADP (from 0.05 to 1.25 µmol/assay) was added to the nine assay cuvettes and the volumes made up to 2.95ml with water. After preincubation for 3 minutes at 25°C in situ the reactions were initiated by addition of enzyme (50 µl). The K_mADP values were obtained by the standard regression procedure and are plotted as -log K_m (mM). The assay concentration of PEP was 1.67mM.

Nucleotide specificity

The specificity of the purified enzyme for the nucleoside diphosphate phosphoryl acceptor was investigated, and comparative apparent K_M and V_{max} values are shown in Table 22. The preferential birding of ADP and GDP by this enzyme parallels the behaviour of the muscle enzyme (Plowman & Krall, 1965), although the binding of GDP by the trypanosomal enzyme is relatively stronger with respect to ADP. The primary conclusion from the data on CDP and probably on UDP, is that the apparent K_M values are higher than those of ADP and GDP. The large standard deviation values of the extrapolated K_M and V_{max} values cast some doubt on the validity of the absolute values in the cases of UDP and CDP, chiefly due to the difference between the available range of coenzyme concentrations ($\leqslant$ 2mM coenzyme) and the apparent K_M values.

pH dependence of the apparent Michaelis constant for ADP.

Direct involvement of thiol groups in the action of PK is difficult to demonstrate. It was hoped that the ionisation of one or more of these groups could be shown to affect the kinetic parameters of the enzyme, but unfortunately the extremely low yields obtained from the purification and the low rates of activity in the normal ionisation range of thiol groups precluded this demonstration. To avoid the discontinuities often shown in enzyme responses to change in pH where different buffers are used in different regions of the pH range, the method described in Figure 24 was used to determine the effects of pH on the K_MADP value, with the results shown. The strict interpretation of these data in terms of the ionisation of groups on the enzyme, ADP or enzyme-ADP complex is limited due to the unfeasibility of using saturating concentrations of PEP for every assay. The S_{50}^{PEP} value for the enzyme in the purified state is approximately

1 mM-PEP, a figure so high that saturation of the enzyme requires prohibitively high substrate levels. Despite this difficulty, the inflection in the pK_M versus pH graph is interpretable qualitatively in the following manner.

The graph of pK_S versus pH theoretically consists of straight-line sections of integral slope, joined by short, curved, corners. Each inflection in the graph indicates the ionisation of a group in one of the reaction components, the pK_a being derived by extrapolation of the linear segments to their intersection. The ionisation of a group in the EX complex (where X is a substrate or cosubstrate) produces a unit increase in the slope; that of a group in either the free enzyme or substrate produces a unit decrease in the slope. Quantitatively, the distance between the linear intersections and the curvature of the graph is equal to a (vertical) distance of log 2 = 0.3; if two groups ionise at the same pH, this distance is equal to log 3. Due to the limitation imposed upon these data by the use of a non-saturating concentration of PEP this quantitation is not permissible in this case.

When the ionising group on the enzyme or the free substrate is directly involved in the binding of enzyme and substrate, the ionisation is normally entirely suppressed and results in a discrete kink in the graph (cholinesterase, Dixon, 1953: alkaline phosphatase, ibid.,). The wave-like form as seen in the case of this trypanosome enzyme, has been interpreted as the ionisation of a group not actively involved in the complexing of enzyme and substrate, but one whose pK_a is altered by this combination (Dixon & Webb 1958); in this case the pH shift is from approximately 6.6 in the free enzyme or substrate to 6.1 in the EMgADP complex. The titration curve of ADP in the presence of the assay concentration of Mg^{2+} ions shows no ionisation in this region, indicating that this ionisation is due to an enzymic group at or near the enzyme active site.

The question naturally arose as to the relative stability of the enzyme over this pH range, and the following data indicate that this is not a significant factor. Two assay mixtures were prepared at pH 6.02 and 7.21 respectively, in a final volume of 2.80 ml. Enzyme (100 µl) was added to each of these media, and after 2.5 minutes the acid assay system was brought to pH 7.21 $\pm$ 0.05 by addition of 0.1 M-KOH.

Both experimental and control incubations were started by addition of ADP (1.25 µmole) and PEP (5 µmole) at six minutes, and the initial velocities were measured. This experiment was repeated with a sample preincubated at pH 7.71, and retitrated with 0.1 M-HCl to pH 7.21, the following enzymic rates being obtained after correction for the volume changes on titration:

	Rate (µmoles/5 minutes)
pH 6.02	0.287 $\pm$ .024
Standard	0.312 $\pm$ .011
pH 7.71	0.304 $\pm$ .026
Standard	0.308 $\pm$ .015

The comparatively large errors in these figures arise from the difficulty of accurately measuring the final volumes of the titrated experimental assays, but some indication is obtained that the profiles obtained for variation of the kinetic parameters over this pH range are real, and are not artefacts due to the increased instability of the enzyme at the extremes of pH.

Effect of PEP concentration

The activating effect of PEP on this enzyme, which was suspected from the work carried out on the crude material, was confirmed in a considerably more reproducible fashion on the purified material. The direct and

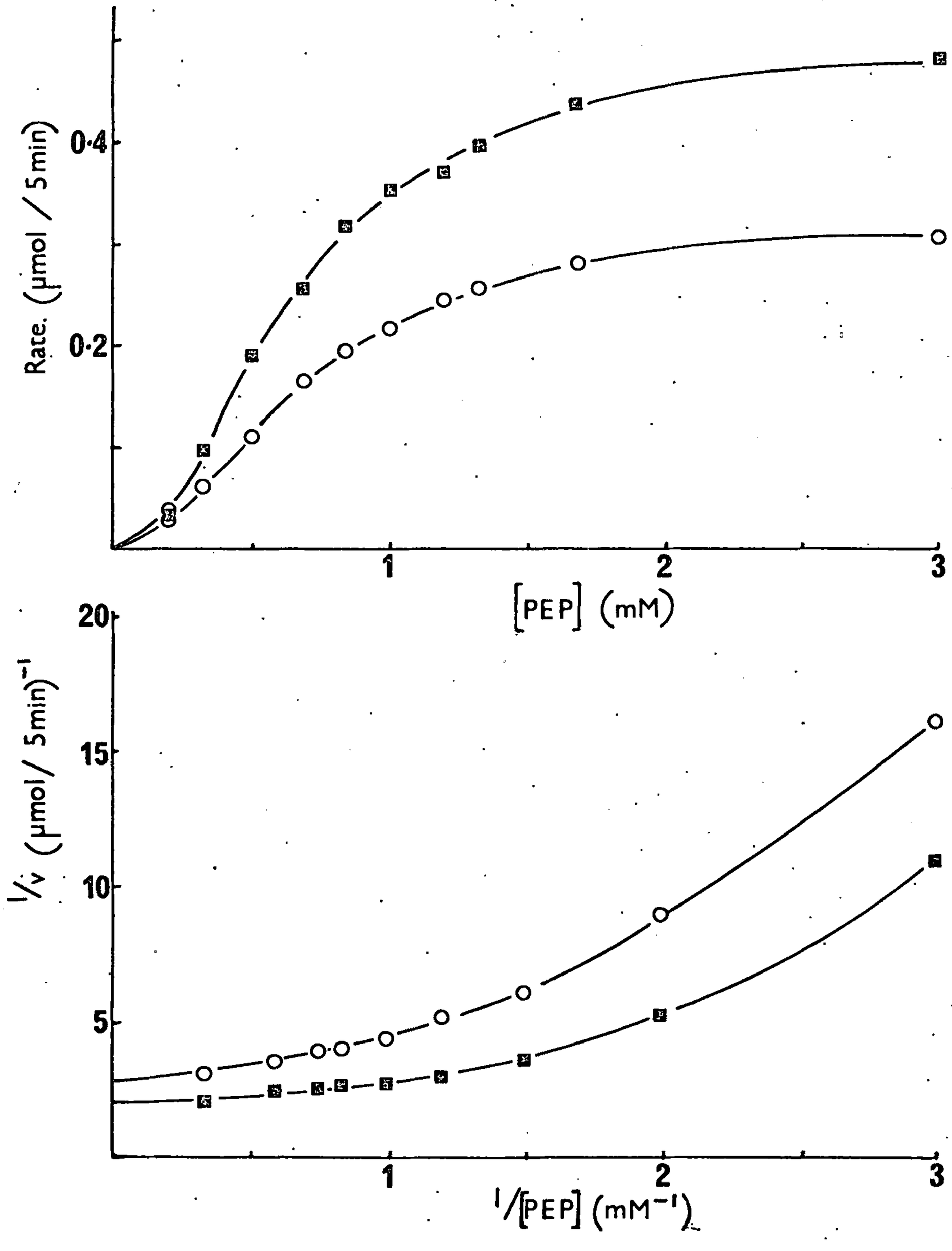


Fig. 25. The effect of PEP concentration on the activity of purified pyruvate kinase: direct and Lineweaver-Burk plots. The data were obtained as for Fig. 22. The concentration of ADP was ■ = 4.17×10^{-4}M. ○ = 0.83×10^{-4}M.

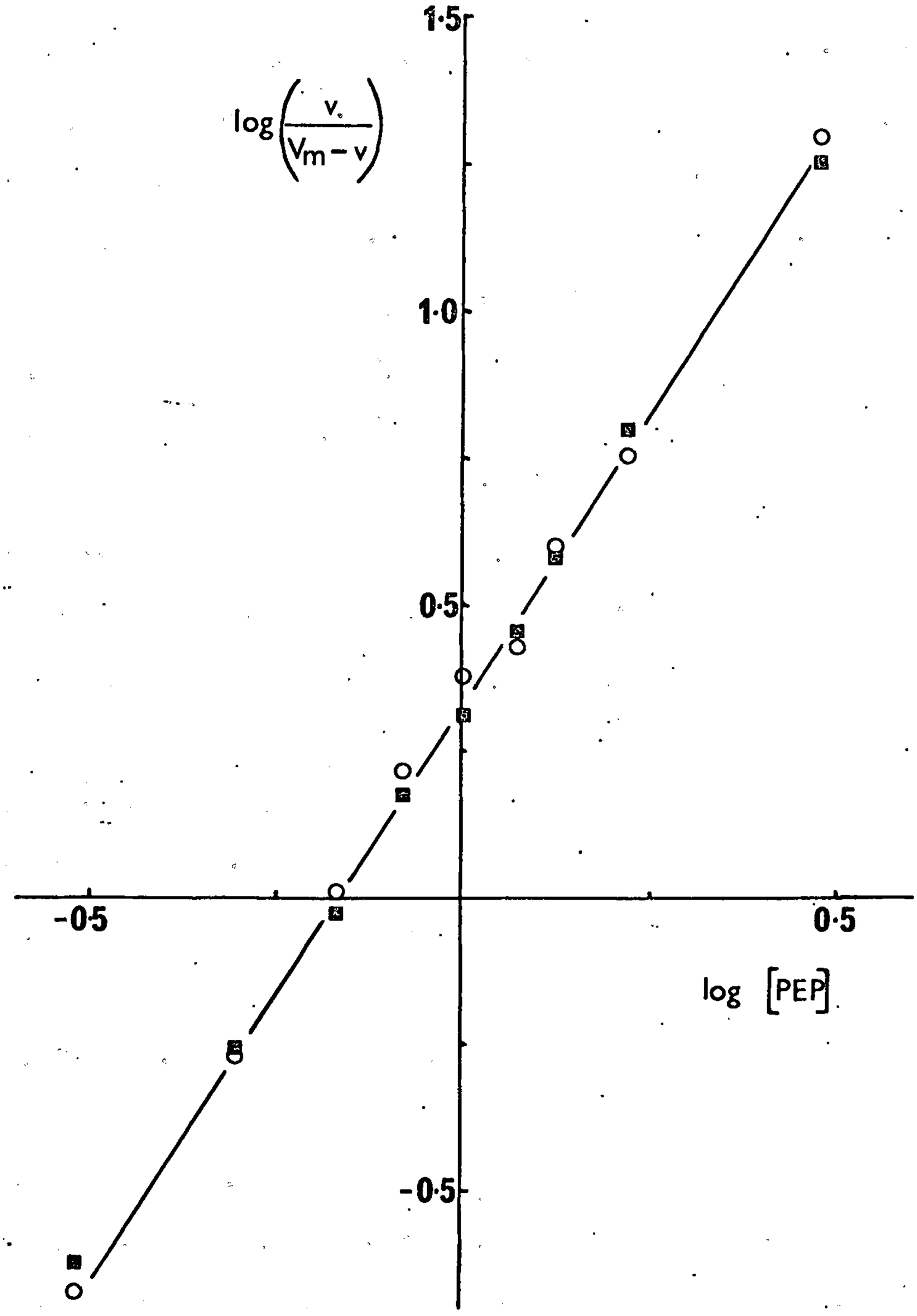


Fig. 26. The effect of PEP concentration on the activity of purified pyruvate kinase: Hill plots. The data of Fig. 25 expressed as the double logarithmic plot. Analysis of the data was carried out as described in Chapter 3. ■ = 4.17×10^{-4}M-ADP; ○ = 0.83×10^{-4}M-ADP.

reciprocal plots in Figure 25 are illustrations of the type of data obtained by variation of the PEP concentration and are included solely as an example of the forms of the curves. These methods of graphical analysis are of little kinetic use, and the same data are replotted in Figure 26 in the form of Hill plots.

It may be seen that variation of the coenzyme concentration over the range 8.3 - 41.7 x 10^{-5} M-ADP does not alter the kinetic parameters of the enzyme with respect to PEP. This independence was confirmed by repetition of these measurements on two other enzyme batches.

Figures 25 and 26 show an S_{50} value of 0.69 mM-PEP. Subsequent purified batches, when assayed immediately after the final stage of purification, yielded S_{50} values ranging from 0.82 - 2.31 x 10^{-3} M-PEP a considerable range of estimates. From eleven purified specimens, the mean and S.E.M. of the S_{50} PEP value was 1.44 $\pm$ 0.13 x 10^{-3} M-PEP.

The sigmoidal shape of the substrate concentration versus velocity curve, indicates the possibility of allosteric control of this enzyme, although many other kinetic conditions could account for sigmoidicity of this nature. Under the standard conditions of assay employed in these experiments, linearity of the Hill plot over the entire experimental range of substrate concentrations is obtained. However, in some subsequent experiments, where optimal conditions no longer hold and much lower rates were obtained, non-linearity at the extremities of the graph was observed. In these cases, the gradient through the abscissa, where the concentration of PEP = the S_{50} value, is used to define the Hill coefficient.

pH dependence of the kinetic parameters of PEP utilisation

The experiments defining the effects of pH change on the S_{50}PEP, V_{max} and n_{PEP} values of the enzyme, were carried out in the same manner as those

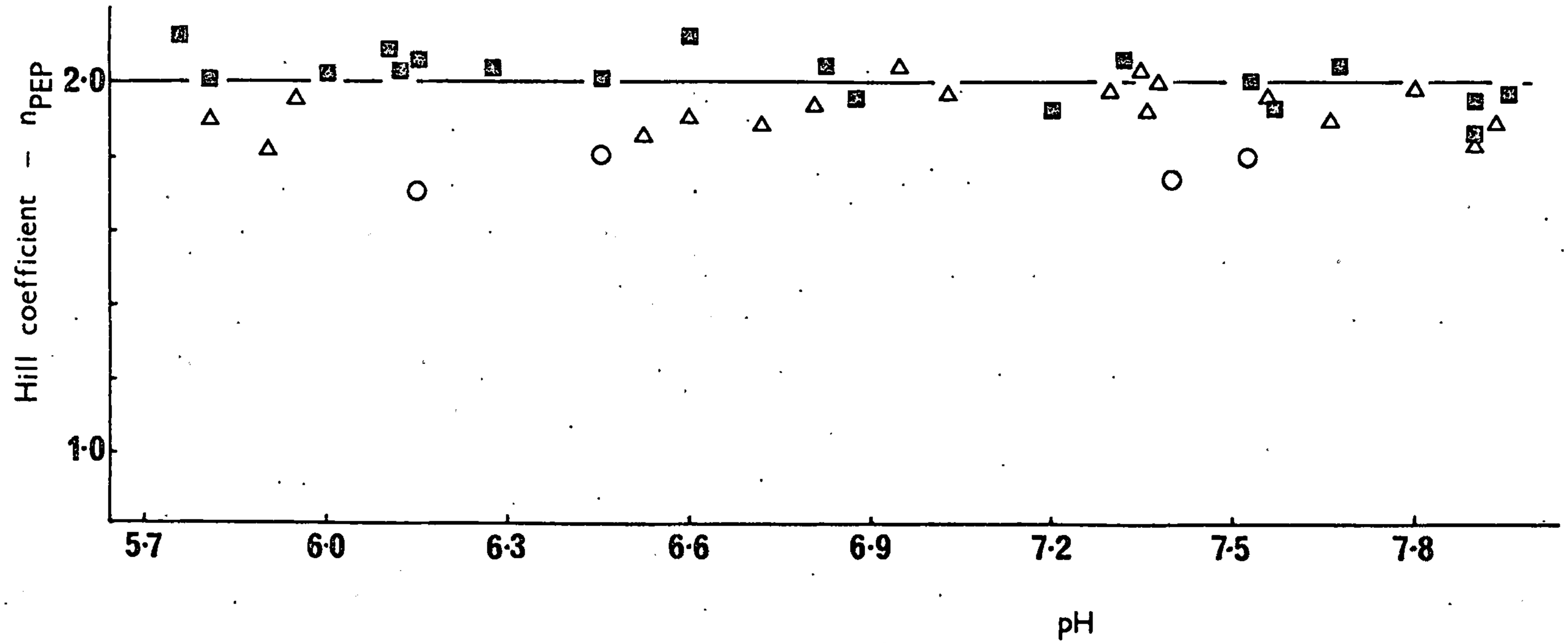


Fig. 27. The effect of pH on the Hill coefficient for PEP of purified pyruvate kinase. Initial velocity data were obtained as described in Fig. 24, and the Hill coefficients calculated as described in Chapter 3. Two preparations of enzyme were used as follows. Δ = preparation 1. ▣ = preparation 2 less than 48 hours after purification. O = preparation 2 72 hours after purification. Preparation 2 had an initial V_{max} value of 2.81 μmol/min/ml enzyme at pH 7.20 and was diluted with TGD to equal that of preparation 1 (2.09 μmol/min/ml). ADP concentration = 1.25 mM.

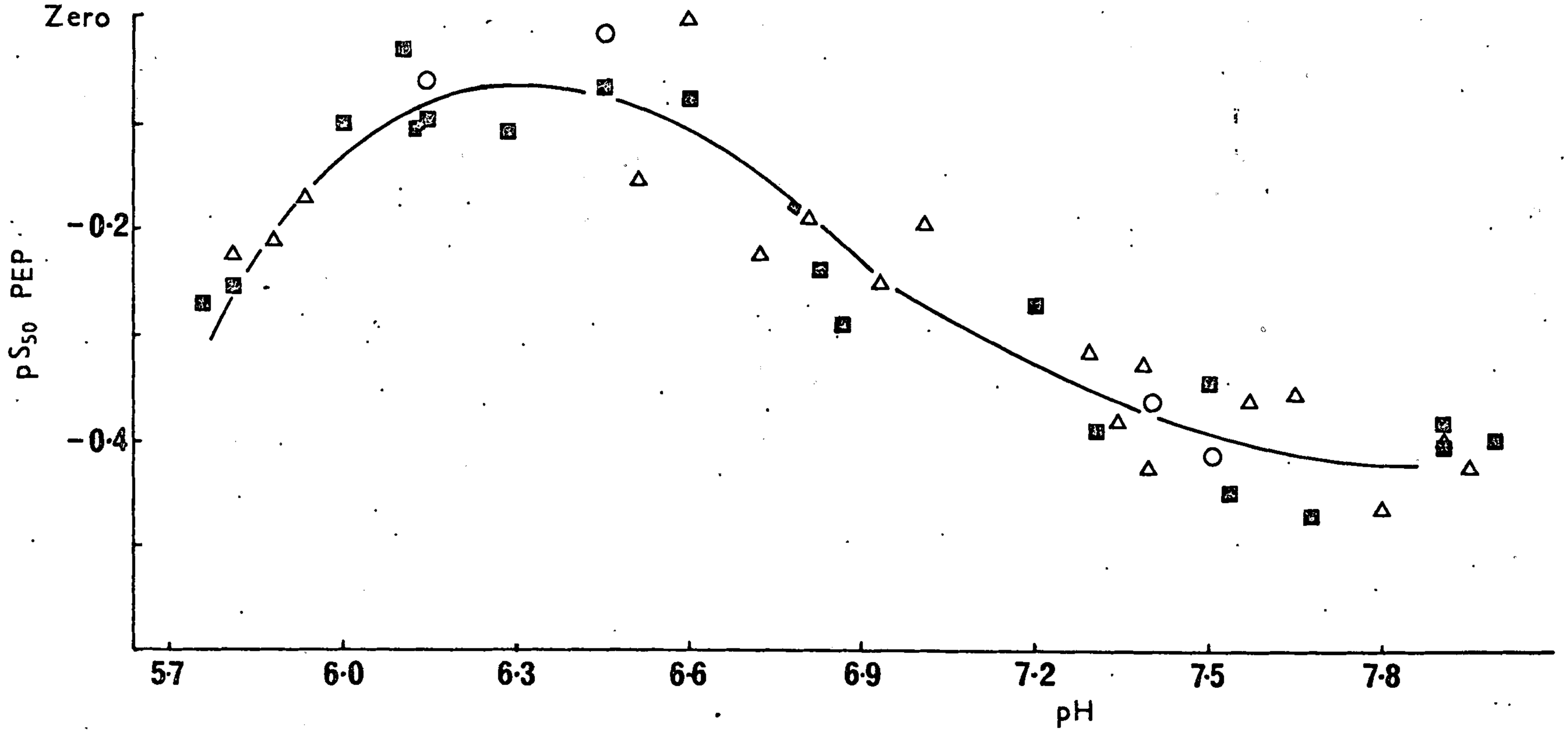


Fig. 28. The effect of pH on the S_{50}PEP value of purified pyruvate kinase. Data are from the same experiments as Fig. 27. S_{50} values were expressed as mM-PEP and plotted as -logS_{50}. ADP concentration = 1.25 mM.

in which the variable was the ADP concentration. The pH range available for study was again limited due to the extremely low rates of reaction obtained outside the extremes of pH 5.8 and 7.9, and the data obtained are expressed in Figures 27-29.

i. The Hill coefficient.

Over the pH range which was investigated, the cooperative interaction of the multiple PEP binding sites appears to be unchanged, in marked contrast to that of the liver L type enzyme, where the coefficient rises continuously from n = 1.0 at pH $\leqslant$ 6.8 to n = 3.0 at pH 8.5 (Rozengurt et al., 1969).

ii. The S_{50} value.

These data, which are analysed graphically in the form of the pS_{50} ($-\log S_{50}$) values to simplify interpretation, are subject to the same limitations as mentioned for the variation in K_M ADP with pH. The slopes of the extrapolated linear parts of this plot are theoretically integral, each ionisation producing a change of $\pm$ 1 in the slope. As in the neighbourhood of the pK value, the enzyme or substrate group contributing to the ionisation is not wholly in either of the ionic forms available, curvature of the experimental data is expected, and the linear gradient between pH 6.9 - 7.4 must be interpolated. Depending upon the number of points included in the determination of the regression line of slope -1 in this pH region, different values for the two pK_a's (defined by the intersection points) are obtained as follows.

Inclusive pH values used in regression	pK_a values obtained a)	b)
6.45 - 7.51	6.81	7.17
6.60 - 7.68	6.93	7.28
6.71 - 7.20	6.75	7.11

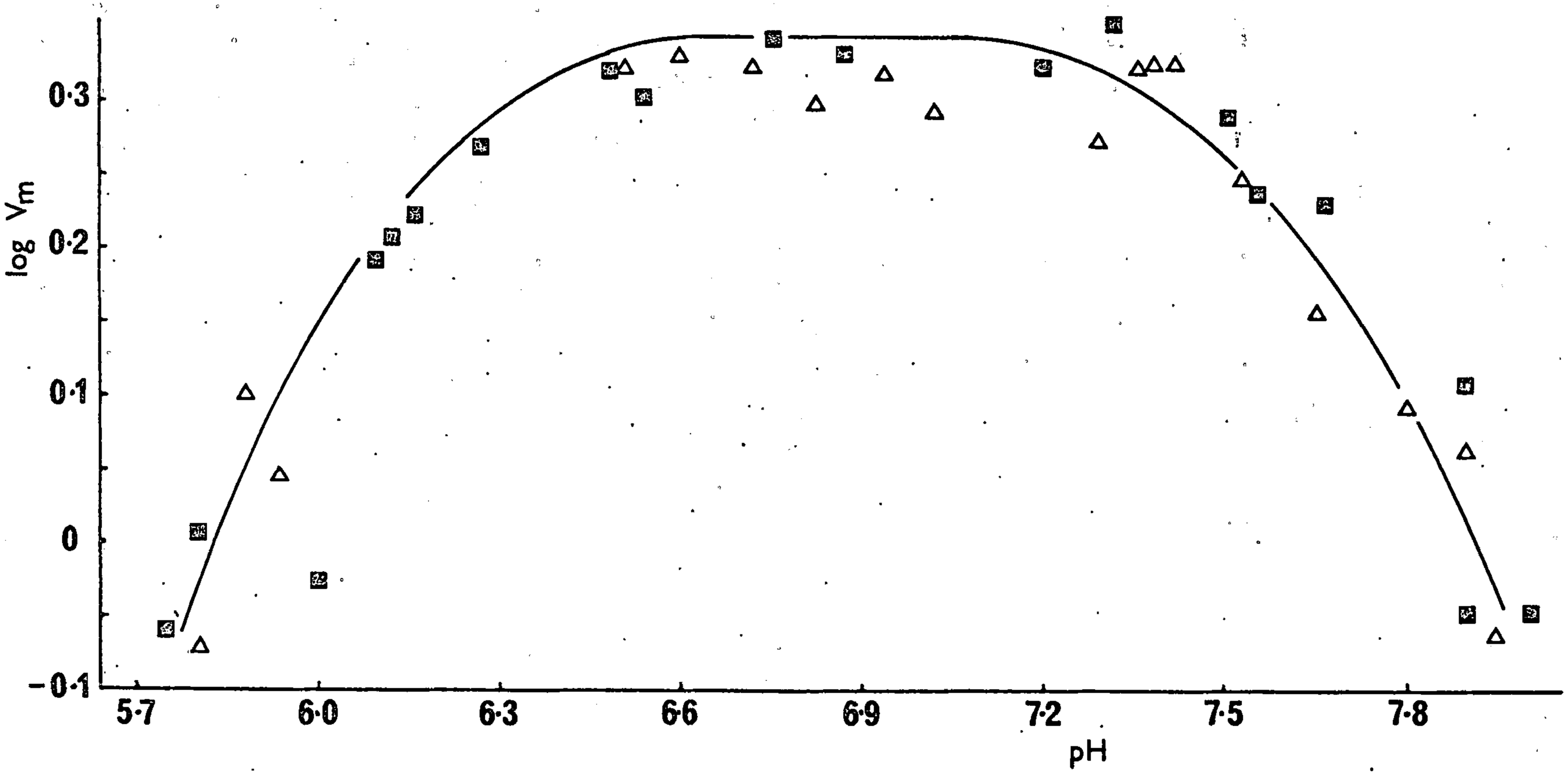


Fig. 29. The effect of pH on the V_{max} value of purified pyruvate kinase. Data are from the same experiments as Fig. 27. V_{max} values were expressed as µmol/min/ml enzyme and are plotted as log V_{max}. All values are normalised to the figure at pH 7.20 as described in the adjacent text, to allow for the instability of the enzyme. ADP concentration = 1.25 mM.

The directions of inflection define the group with the higher pK as belonging to the enzyme-substrate complex, and the one ionising in the lower range as being on either the enzyme or the substrate. Again however, titration of PEP in the presence of Mg^{2+} ions showed no ionisation of this substrate in the range pH 6.2 - 8.0, indicating that the group resides on the enzyme molecule. It is not possible to decide whether these pK_a values describe two separate groups, or the ionisation of one group whose pK_a shifts on binding PEP, from the lower to the higher range. The third ionisation apparent from these experiments, at pH 5.9 - 6.0, corresponds to the ionisation of PEP which occurs at pH 6.0 in the presence of Mg^{2+} ions. It thus appears that a doubly ionised phosphoryl group is essential for the binding of PEP to the enzyme.

iii. <u>The V_{max} value</u>.

Under the conditions at which V_{max} obtains, the enzyme is totally saturated with substrate; in this case where cooperativity exists between binding sites for PEP, the enzyme is totally in the ES_n state (where n molecules of PEP are bound per enzyme molecule). Any effect of a variation in pH will therefore reflect an ionisation of the ES_n complex, effects on the affinity of the enzyme for PEP being eliminated.

Over the period required to perform these experiments ($2\frac{1}{2}$-3 days), the V_{max} value of the enzyme drastically decreases, in terms of /umoles / minute / ml enzyme. Hence the data have been normalised with respect to the V_{max} value obtained immediately after purification at pH = 7.2. Repeated estimation of the V_{max} value at this pH enabled a correction to be made for the data at other pH values. Figure 29 indicates that two groups in the ES_n complex ionise in the pH range 5.7 - 7.9, with pK_a values of 6.2 - 6.3 and 7.5 - 7.6.

It is possible that the group ionising at pH 7.5 - 7.6 is identical with the ionisation estimated at pH 7.1 - 7.3 from the graph of pS_{50} *v.* pH. This would indicate that the real value of the ionisation lies at the upper end of the 7.1 - 7.3 pH range, thus defining the position of the other pK_a on the enzyme as being at the top of the estimated range of pH 6.7 - 7.0.

The value of pK_a = 6.2 - 6.3 in the ES_n complex is not reflected in a similar inflection in the pS_{50} *v.* pH profile, thus involving this group in one of two functions. Firstly, it may be catalytically required, but not involved in the binding of PEP to the active site. Alternatively, as the S_{50} value is a catalytic constant and not an allosteric constant, the group may be involved in the binding of PEP to the allosteric site. The lack of effect of pH on the Hill coefficient for PEP would suggest the former explanation as being correct. These data may be summarised as follows.

		pK_a of dissociating group			State required for maximal activity
	Derivation	Enzyme	ES	PEP	
(1)	V_{max}		6.2-6.3		dissociated
(2)	V_{max}		7.5-7.6		undissociated
(3)	pS_{50}		7.2-7.3		undissociated
(4)	pS_{50}	6.7-7.0			undissociated
(5)	pS_{50} and titration of PEP			5.9-6.1	dissociated

with the provisos a) that (2) and (3) may refer to the same group and b) that (3) and (4) may refer to a single group whose pK_a shifts on binding substrate.

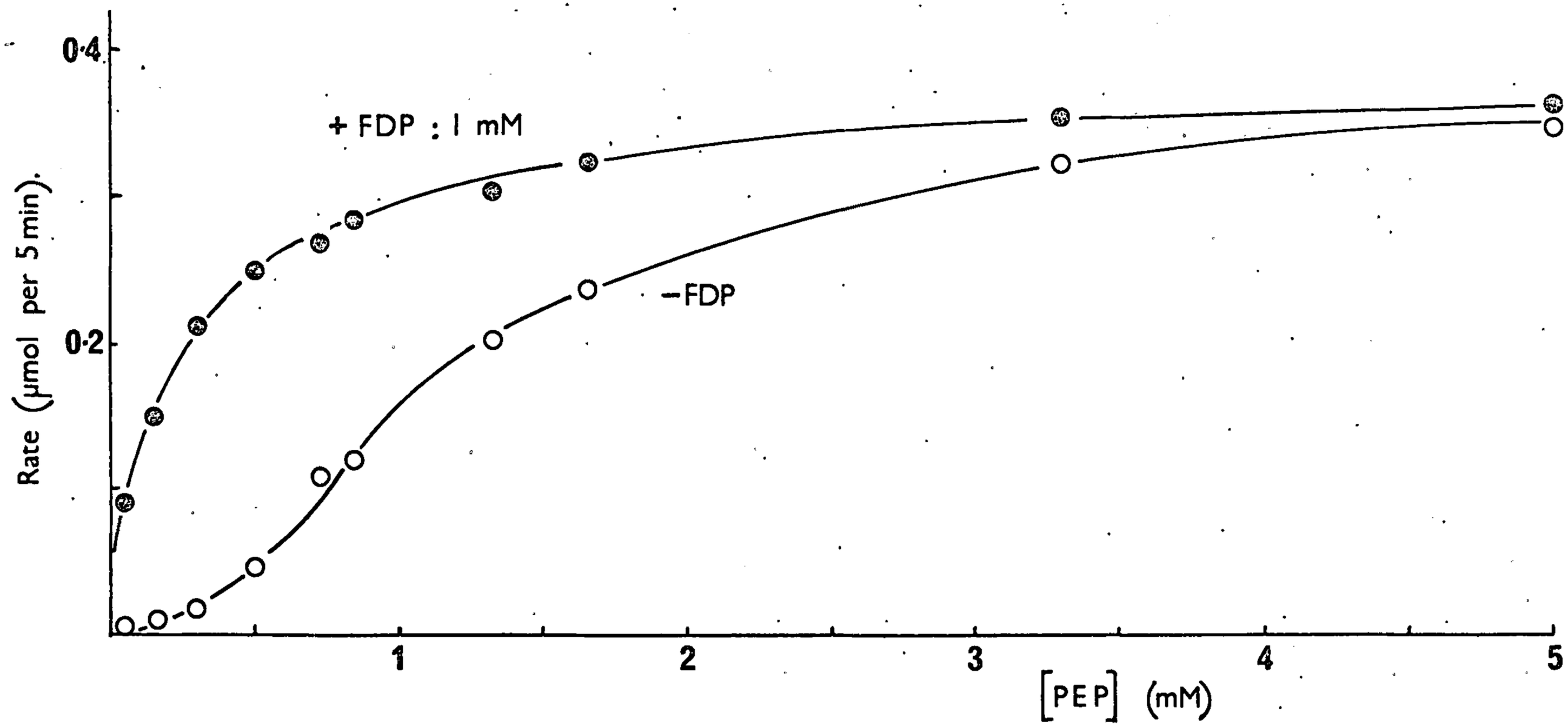


Fig. 30. The effect of FDP on the response of purified pyruvate kinase to PEP. Aliquots of purified enzyme were preincubated in the standard assay medium for 3 minutes at 25°C, with and without FDP (1.00 mM). Reactions were initiated by the addition of PEP as indicated and ADP to a final concentration of 4.17×10^{-4} M.

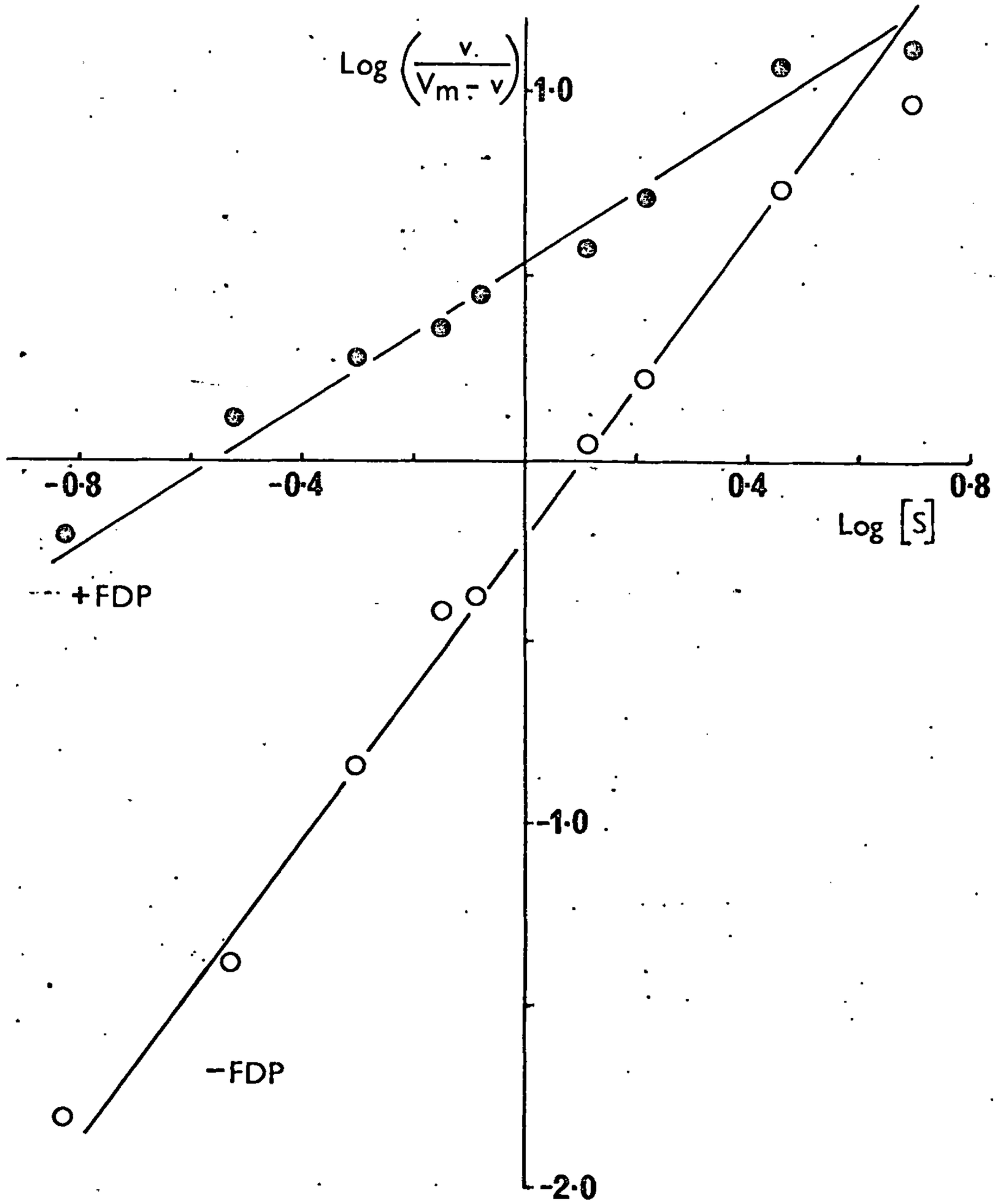


Fig. 31. The effect of FDP on the response of purified pyruvate kinase to PEP. Hill plots of the data of Fig. 30. The concentration of ADP = 4.71×10^{-4} M. The concentration of FDP = 1.00mM.

† In a series of five experiments the Hill coefficient decreased from 2.02 ± 0.04 to 0.99 ± 0.03 and the S_{50}PEP value decreased from 1.34 ± 0.05 mM-PEP to 0.25 ± 0.02 mM-PEP. The ratio Vmax + FDP / Vmax - FDP in the same experiments was 1.01 ± 0.03.

6) ALLOSTERIC CONTROL OF PURIFIED TRYPANOSOME PYRUVATE KINASE.

i. Effects of FDP

The activation of a pyruvate kinase by FDP was first reported by Hess et al., (1966) for a liver enzyme, and has since been found to obtain in yeast and in other sources of the enzyme (e.g. Gancedo et al., 1967; Taylor & Bailey, 1967). The effects of high concentrations of FDP (10^{-3}M) on the trypanosomal enzyme are exemplified in Figure 30, although this apparent normalisation of the sigmoidal response to increasing PEP concentration, is not by itself significant. As pointed out by Atkinson (1966), a subjective evaluation of apparent differences of this nature may lead to the wrong conclusions, as a contraction of the curve along the abscissa leads to a curve which superficially resembles the normalised hyperbola. To demonstrate a loss of cooperativity between substrate molecules, recourse must be taken to analysis by the Hill plot, and these data are redrawn in terms of log $\frac{v}{V_M - v}$ versus log (S) in Figure 31. Here it may be seen that the presence of 10^{-3} M-FDP has two effects on this enzyme. It abolishes the cooperative interactions of PEP, the Hill coefficient decreasing from 2.12 to 0.91, and it decreases the S_{50} PEP value from 1.20×10^{-3} M-PEP to 0.25×10^{-3} M-PEP. No change in the V_{max} value of the system is apparent.†

These assays were carried out with a preincubation period of three minutes in the presence or absence of FDP, prior to the reaction being started by addition of ADP and PEP at this time. A greater gross activation is obtained under these conditions than with no preincubation period, where the reaction is started by addition of enzyme. This, however, is more likely to be due to an increase in the stability of the enzyme in the presence of FDP, than to a time-dependent allosteric transition. However, large increases in activity are found in the presence of FDP, when the reactions are started by addition of enzyme.

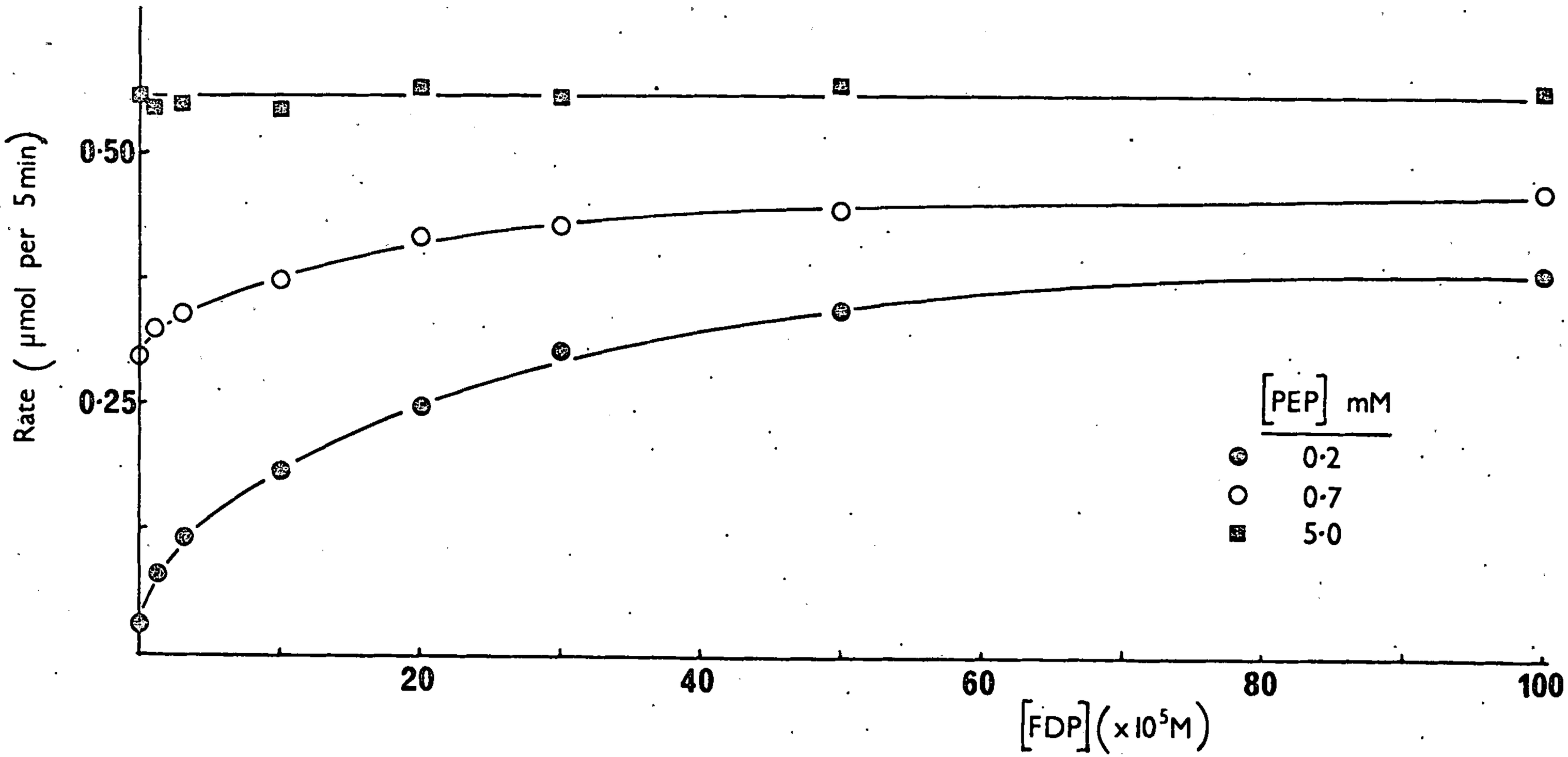


Fig. 32. The effect of PEP concentration on the activation of purified pyruvate kinase by FDP. Aliquots of enzyme were preincubated in the standard assay medium at various concentrations of FDP for 3 minutes prior to initiation of the reactions by the addition of ADP (4.17×10^{-4} M) and PEP as indicated.

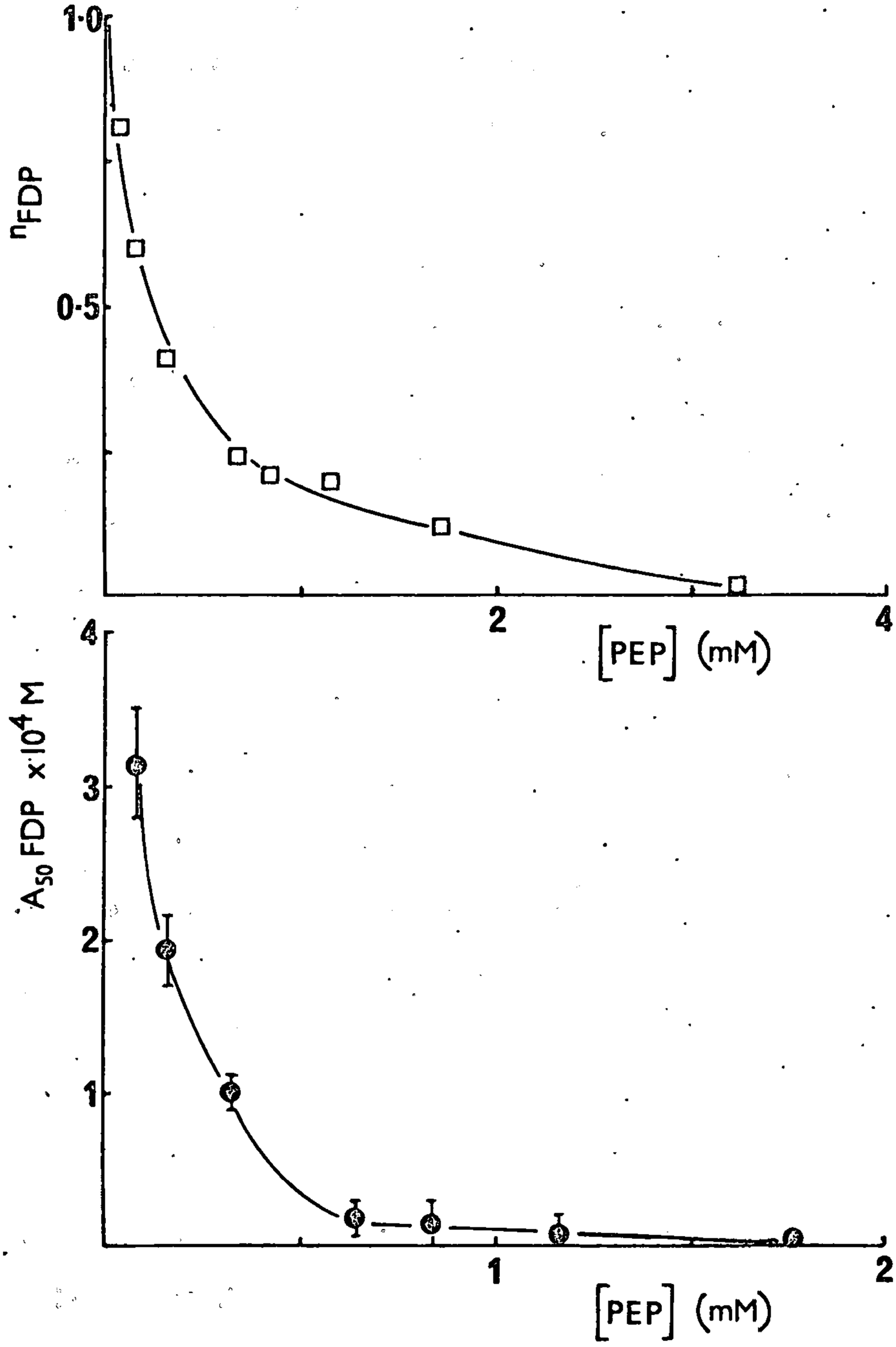


Fig. 33. The effect of PEP on the binding of FDP to purified pyruvate kinase. Experimental data were obtained as for Fig. 32. Hill coefficient and A_{50}FDP values were obtained from Hill plots as described in Chapter 3.

The activity of FDP in producing a hyperbolic response to PEP concentration, mimics the effect of allosteric binding of the substrate, as in the latter part of the sigmoid, unactivated plot, normalisation has, in effect, occurred. Thus it is not surprising that the activation by FDP is dependent upon the concentration of PEP, as further shown in Figure 32.

When the concentrations of substrate and activator are varied independently it is found that PEP and FDP act entirely in conjunction in activating the enzyme. Modified Hill plots for FDP yield activator constants (A_{50} values) and Hill coefficients for FDP as shown in Figure 33. By extrapolation to a zero concentration of PEP, it is found that one molecule of FDP only is bound under these conditions (n = 1.0), or alternatively, that no interaction is occurring between multiple FDP binding sites. On the other hand, as the concentration of PEP decreases, the A_{50} value for FDP (i.e. the concentration of FDP required to give 50% the maximal velocity at a constant PEP concentration) increases.

The question arose as to whether the primary effect of FDP is to decrease the Hill coefficient for PEP, or to decrease the S_{50} value of this substrate. The result of the variation of effector concentration on these two kinetic parameters are demonstrated in Figure 34. Up to concentrations of FDP in the region of 10^{-4} M, the major effect is to decrease the interaction between substrate binding sites, this action being reflected in the decrease of the Hill coefficient from 1.94 to 1.01. Over the same range of FDP concentrations, the S_{50} PEP value is decreased slightly from 1.21 mM to 0.96 mM, a drop of 20%; higher concentrations of the activator rapidly produce a much more substantial increase in the affinity of the enzyme for PEP, the S_{50} value decreasing to 0.32 mM, a drop of a further 53%.

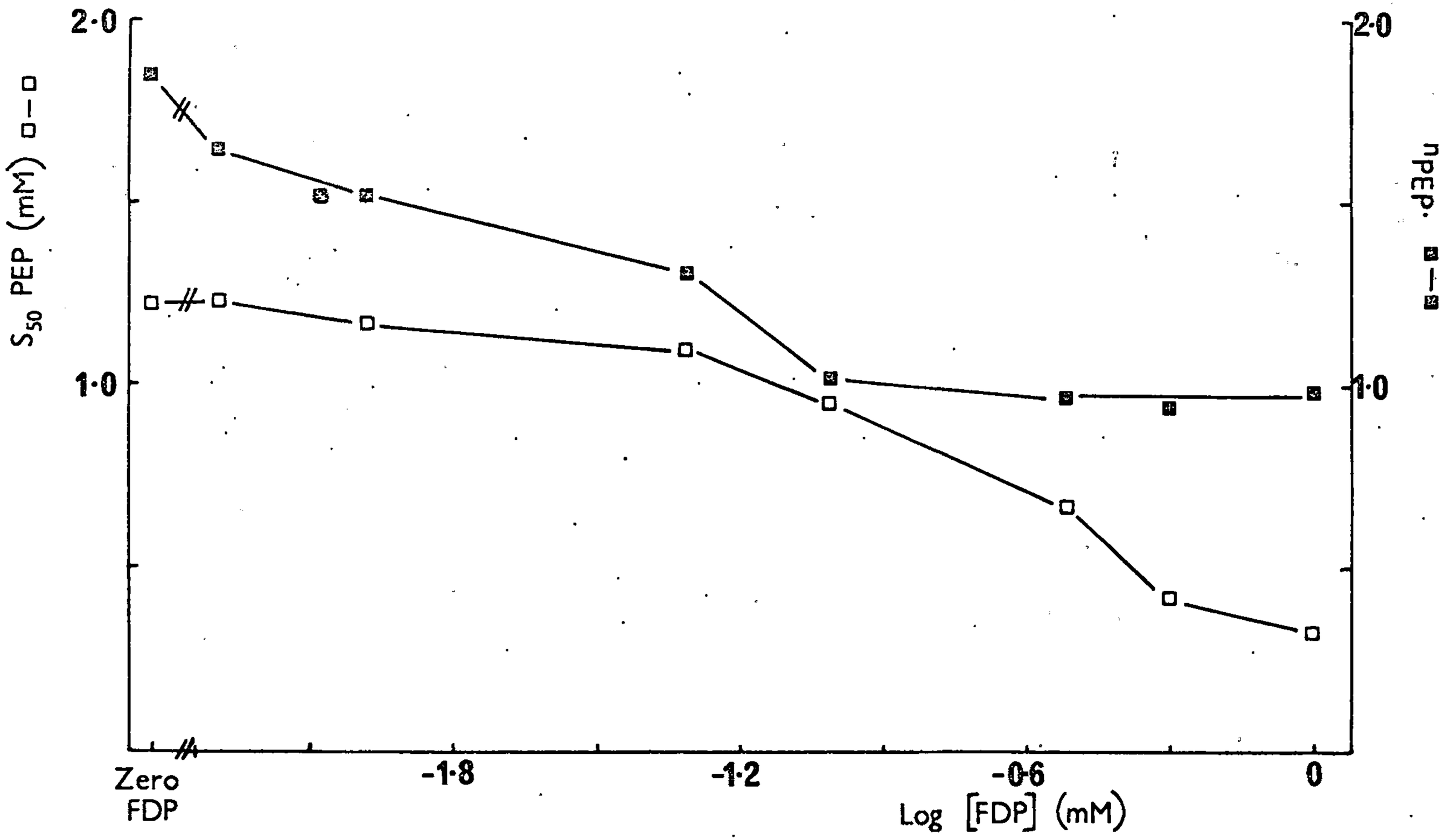


Fig. 34. The effect of FDP on the kinetic parameters of PEP utilisation by purified pyruvate kinase. Hill coefficients (n) and S_{50}PEP values were obtained at different FDP concentrations as described in Figs. 30 & 31, with a preincubation period of 3 minutes and a concentration of ADP of 4.17×10^{-4}M. Each point is from a set of at least eight concentrations of PEP from 0.5 to 3.33 mM.

The activation of the enzyme by FDP appears to be a property unique to this glycolytic intermediate. No effect of the following compounds was found, when the enzyme was assayed after three minutes incubation in the presence of the metabolite, at a PEP concentration of 0.67 mM.

Metabolite	Concentration
Fructose	10^{-3}M
Glucose	5×10^{-3}M
Glucose-6-phosphate	10^{-4}M
Fructose-6-phosphate	10^{-4}M
3-PGA	10^{-3}M
2-PGA	10^{-3}M

When the effect of FDP on the K_M value for ADP was investigated, no effect on this parameter was found up to a concentration of FDP of 10^{-3} M. The activation appears, therefore, to specifically affect the binding of PEP.

ii. Effects of ATP.

Allosteric control by ATP of PK from many tissues has been reported (e.g. liver, Tanaka et al., 1967b) and this effector is thought to work in an antagonistic manner with the activation of the enzyme by FDP. However, in some cases, this inhibition by ATP has been shown to be effected simply by chelation of the required divalent cation by the nucleoside triphosphate, thereby decreasing the concentration of $MgADP^-$ available to the enzyme (Wood, 1968). On the basis of the formation constants of $MgATP^{2-}$ and $MgADP^-$ (Melchior, 1965), Wood showed that many earlier reports of ATP inhibition could be accounted for in this manner without invoking the necessity of an allosteric binding of ATP. This led to the introduction by Boyer (1969) of a Mg^{2+} ion "buffer" system in the

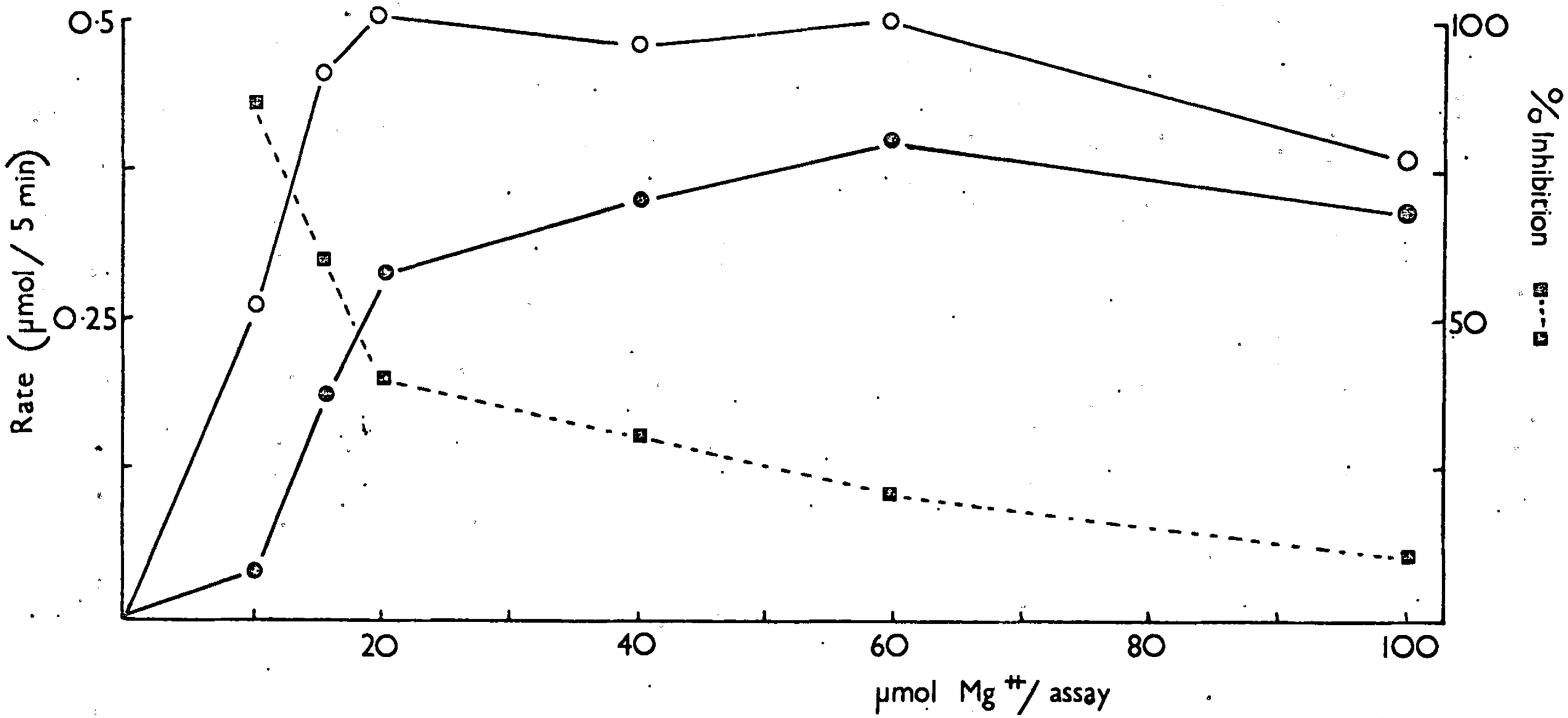


Fig. 35. The effect of magnesium ion concentration on ATP inhibition of purified pyruvate kinase. Aliquots of enzyme were preincubated in the standard assay system for 3 minutes with DL-α-GP (0.05 M), ATP (1.67 mM) and various concentrations of magnesium sulphate. Reactions were initiated by addition of ADP (4.17×10^{-4} M) and PEP (1.67 mM).

form of a large excess of L∝GP included in the assay system. The relative concentrations of the three Mg^{2+} binding substances, L∝GP^{2-}, ADP^{3-} and ATP^{4-} then ensure that up to a total nucleotide concentration of 5 mM, all the ADP^{3-} present in the assay remains as the monomagnesium salt, when the total Mg^{2+} concentration is 30 mM.

During the early stages of this investigation, glucose-6-phosphate was utilised as a Mg^{2+} buffer, but the results obtained were variable and inconclusive. With the publication of the work by Boyer (1969), the buffer was changed to DL∝GP, and more consistent results were obtained. The presence of 0.05 M-DL-∝-GP in the assay system had an inhibitory effect on the enzyme, partly, but not entirely, due to the presence of sodium counter-ions. The contribution of the glycerophosphate anion to this inhibition was, however, only 15% at 50 mM-DL∝-GP and therefore is thought to have little physiological significance, and was not investigated further.

The inclusion of 0.05 M-DL-∝-GP in the assay system with ATP (1.67×10^{-3}M) allowed the effects of the latter compound to be estimated at different levels of Mg^{2+} and ADP. At low Mg^{2+} concentrations e.g. 6.7×10^{-3}M as used in the standard assay system, ATP markedly inhibited the enzyme. However, on increasing the cation concentration, the inhibition decreased steadily to 13% at 33.3×10^{-3} M-Mg^{2+} (Fig.35). The slight stimulation of the enzyme by ATP found at high Mg^{2+} concentrations ($\geqslant$ 67 mM), was due to contamination of the commercial ATP preparations with traces of ADP.

Thus, at these concentrations of PEP and ADP (1.67 and 0.42×10^{-3}M respectively) there appears to be no allosteric control exerted by ATP over the trypanosome enzyme. Mechanistically, it was expected that ATP should at least competitively inhibit the enzyme, as a common binding site for the phosphoryl moiety of PEP and the terminal phosphate group of ATP

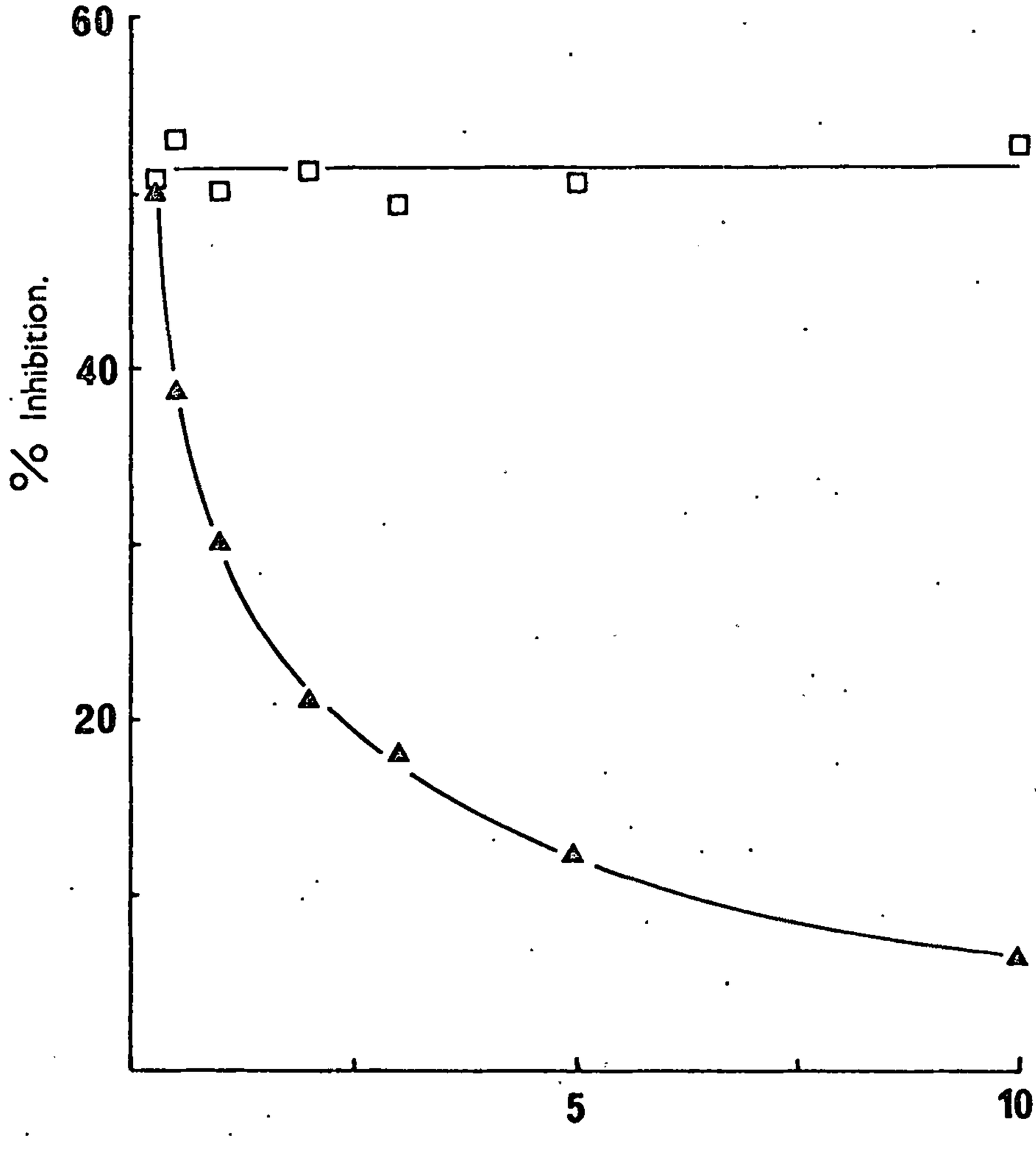


Fig. 36. The effects of EDTA and preincubation time on the melarsen oxide inhibition of purified pyruvate kinase. DTT was removed from the enzyme preparation as described in the text. Aliquots of this enzyme were then incubated in the standard assay medium with and without melarsen oxide for the periods indicated prior to the initiation of the reactions by addition of ADP (4.17×10^{-4}M) and PEP (1.67 mM). □ = +1.00 mM EDTA. △ = no EDTA. Melarsen oxide concentration = 4×10^{-5}M.

as the enzyme product, is commonly postulated. When the substrate and coenzyme levels were simultaneously decreased to 0.5 and 0.1 x 10^{-3}M respectively, this expected effect of ATP was still not apparent, and further decreases in the substrate / coenzyme concentrations were not possible, due to the large amounts of enzyme required to give an accurately measurable rate under these conditions.

7) EFFECTS OF ARSENICALS ON PURIFIED TRYPANOSOME PYRUVATE KINASE

As described previously the instability of the purified enzyme in the assay medium precluded any prolonged preincubation in attempts to ensure equilibrium between the enzyme and the inhibitor. A further complication arose when attempts were made to investigate the inhibitory effects of melarsen oxide in the absence of any stabilising agent, as the drug apparently increases the enzymic stability in the assay medium, as demonstrated in Figure 36. In the presence of EDTA, however, this preferential stability in the presence of the drug disappears.

Under these assay conditions, whereas the I_{50} value of the arsenical was independent of the preincubation time, it was dependent upon both the PEP concentration and the "age" of the enzyme preparation. Freshly purified enzyme preparations gave a mean I_{50} value of 4.3 $\pm$ 0.2 x 10^{-5}M-melarsen oxide (seven estimations) with 1.67 x 10^{-3} M-PEP, which is lower than that obtained with crude material (10^{-4} M). After 24 hours storage in the purified form, the I_{50} figure rose to approximately 1.2 x 10^{-4} M-melarsen oxide and after 72 hours, to approximately 5 x 10^{-4} M-melarsen oxide. All the results reported in this work are from enzymic batches $\leq$ 48 hours after purification.

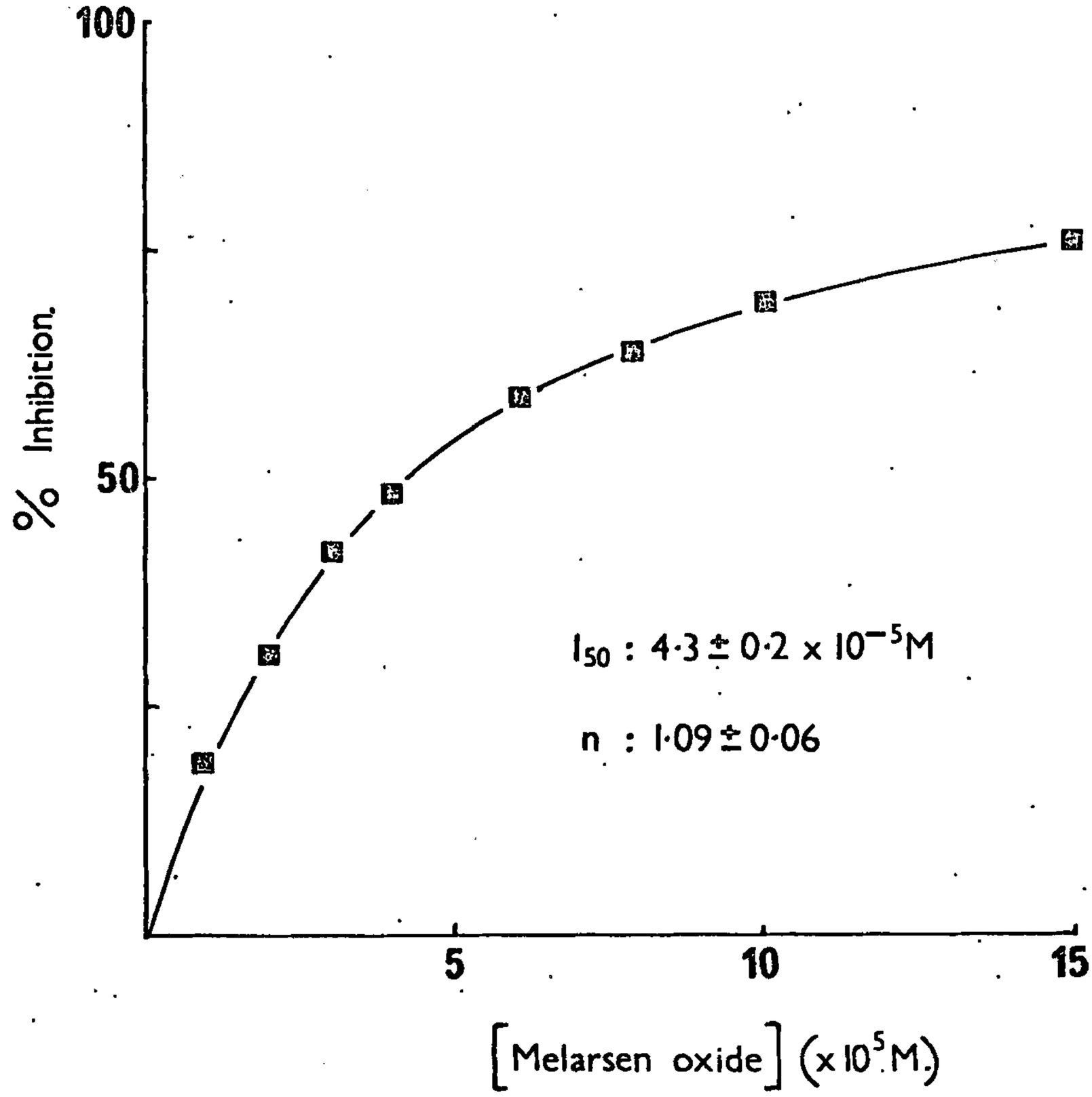


Fig. 37. The inhibition of purified pyruvate kinase by melarsen oxide. DTT was removed from the enzyme as described in the text. Aliquots of this enzyme were then preincubated with various concentrations of melarsen oxide in the standard assay medium plus 1.00mM-EDTA for 5 minutes prior to initiation of the reactions by addition of ADP (4.17×10^{-4} M) and PEP (1.67 mM). I_{50} and Hill coefficient values are derived from the modified Hill plot described in the text.

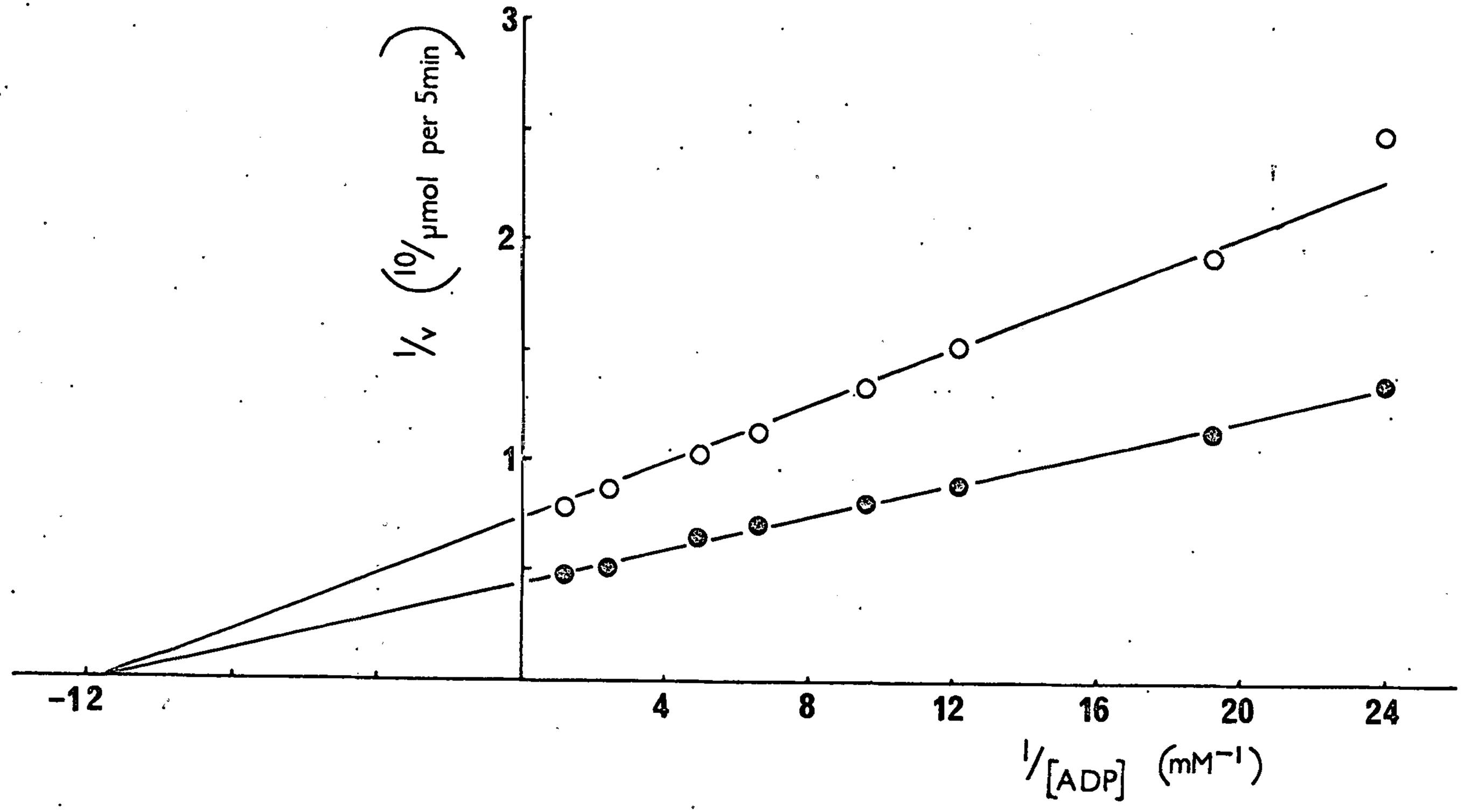


Fig. 38. The effect of melarsen oxide on the kinetics of purified pyruvate kinase with respect to ADP. DTT was removed from the enzyme as described in the text. Aliquots of this enzyme were preincubated with (O) and without (⊘) melarsen oxide (3×10^{-5} M) for 5 minutes prior to initiation of the reactions by addition of ADP and PEP (1.67 mM). Lineweaver-Burk plots were derived as described in Chapter 3. EDTA concentration = 1mM.

No allosteric interaction is found between the arsenical and the enzyme, as may be seen from Figure 37. A double reciprocal plot of percentage inhibition versus melarsen oxide concentration extrapolates to 100% inhibition at infinite inhibitor concentration, and the modified Hill plot of log $\frac{\% \text{ inhibition}}{100-\% \text{ inhibition}}$ versus log melarsen oxide concentration shows a slope of $1.09 \pm .06$. Only one binding site per molecule of enzyme for the inhibitor is therefore indicated with no apparent cooperativity between inhibitor molecules.

Effect of melarsen oxide on ADP binding.

Variation of the ADP concentration in the presence and absence of melarsen oxide, at a fixed PEP concentration of 1.67×10^{-3} M, showed the binding of the coenzyme to be independent of the presence of the drug, as straightforward non-competitive kinetics were obtained (Figure 38). At 3×10^{-5} M melarsen oxide, the K_M value for ADP (i.e. the $MgADP^-$ complex) is not significantly different from the uninhibited value at $8.9 \pm 0.3 \times 10^{-5}$ M, whereas the maximum velocity of the system is markedly diminished in the presence of the drug (from 0.21 to 0.13 µmol / 5 min). Melarsen oxide thus does not interfere with the binding of ADP to the enzyme, but with the catalytic reaction.

Effect of melarsen oxide on PEP binding

In contrast to the non-competitive form of the inhibition by melarsen oxide when the concentration of ADP is the experimental variable, the inhibition obtained with respect to PEP is of a purely competitive nature (Figure 39). The Hill plot of these data indicates that whereas the binding of PEP is altered by the presence of the inhibitor, the S_{50} value increasing from 1.32 mM to 3.23 mM at 10^{-4} M-melarsen oxide, the cooperativity of the system is unaltered. At this inhibitor concentration, the maximum velocity value is similarly unchanged.

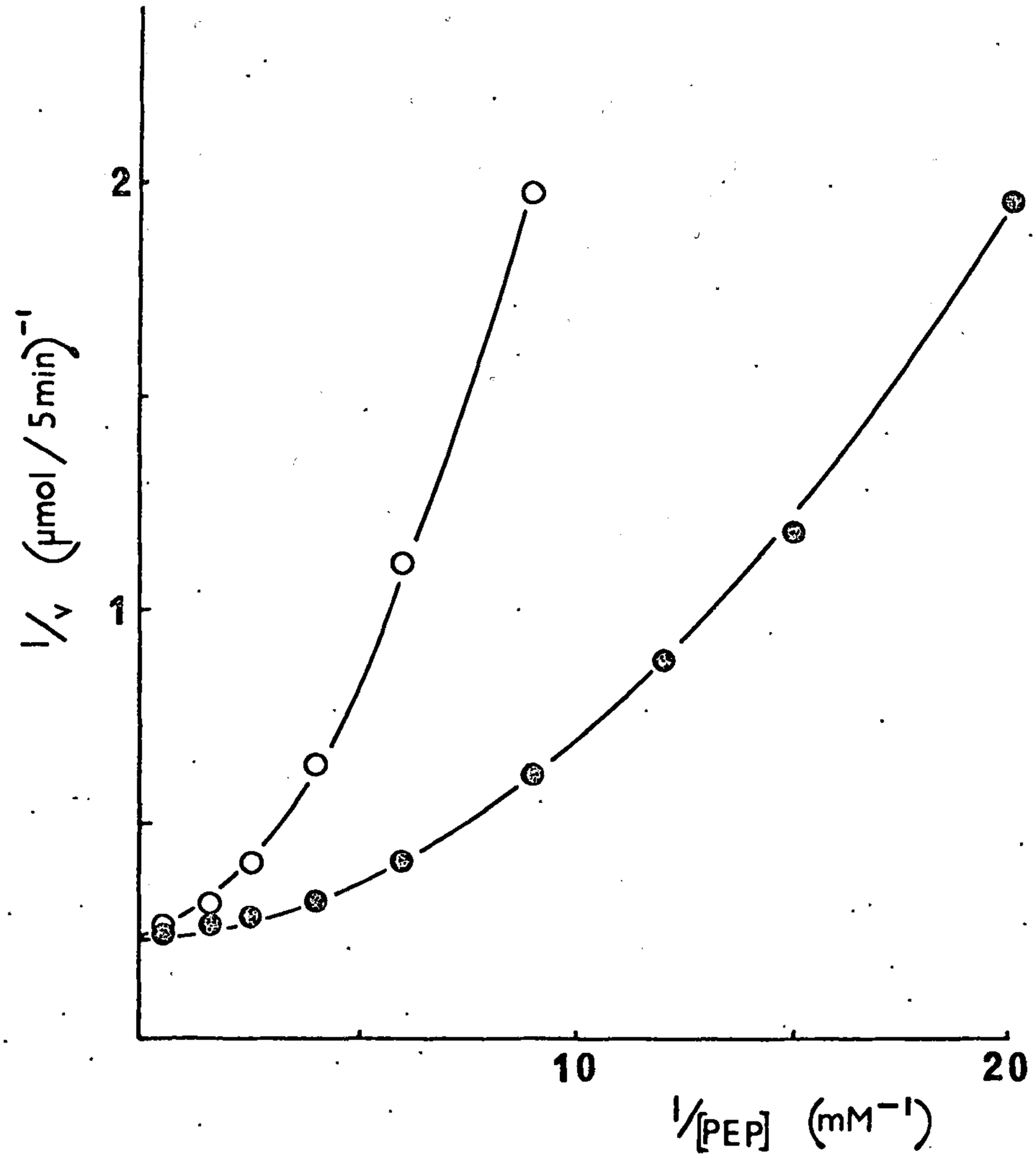


Fig. 39. The effect of melarsen oxide on the kinetics of purified pyruvate kinase with respect to PEP. The concentration of ADP was 4.17×10^{-4} M. All other experimental details were as described in Fig. 38, with the exception that the concentration of melarsen oxide was maintained at 10^{-4} M.

These data were obtained under standard preincubation conditions, in which the enzyme was allowed to interact with the inhibitor for five minutes in the assay medium without ADP or PEP, prior to initiation of the reaction by addition of these cosubstrates. The results presented later in this work regarding the host liver enzyme, and the effects of preincubation time on the mode of inhibition obtained, led to an eventual reconsideration of the trypanosome system. Despite the use of relatively large amounts of the trypanosome enzyme, the inherent instability of the protein limited the available experimental preincubation period to 10-12 minutes; at periods up to this time, the inhibition remained competitive. It is of course questionable as to whether this time period is adequate for the enzyme/inhibitor system to reach an equilibrium position, and attempts were made to enable a longer preincubation period to be used.

Further addition of enzyme to the assay system was not possible, as glycerol, in which the enzyme is stored, protects the enzyme against melarsen oxide inhibition at concentrations above 5%. For the same reason, removal of the glycerol was a fundamental necessity before a batch incubation could be carried out. Dialysis of the enzymic material resulted, in each of three attempts, in the production of a totally inactive residue, presumably due to the prolonged period required for this technique. Elution of the enzyme from a column of G-25 Sephadex with 0.1 M-TEA pH 7.2; 0.1 M-Tris, pH 6.4 or 7.2; 0.1 M-Tris/6.7 mM $MgSO_4$/ 67 mM KCl; or 0.1 M-Tris/0.42 mM ADP^{3-}/0.5 mM PEP, all in the presence of 10^{-3} M EDTA resulted in exceptionally low yields (< 20%) of activity and in even lower stability of the enzyme. In these glycerol-free solutions, the half-life of the enzyme as assayed in the standard medium, is of the order of 15 minutes, if the previously described precautions regarding the degassing of the column are rigorously carried out and the fractions

collected under paraffin oil. This period of time is unfortunately inadequate for the procedure involved in determining the S_{50} value of the enzyme with and without melarsen oxide, and attempts at the removal of glycerol were abandoned. Further attempts at investigation of the effects of prolonged incubation on the interaction of this enzyme and melarsen oxide will only be possible when the purification procedure is scaled up to an extent which will yield large enough quantities of enzyme to allow for the practically total loss of activity on removal of the glycerol.

Interaction of FDP, PEP and melarsen oxide.

The individual effects of FDP, PEP, and melarsen oxide on the purified trypanosome enzyme have been described, but the questions remain 1) is the drug acting as a competitor to PEP at the active or at the allosteric site? and 2) do the properties of the enzyme in the absence of FDP have a real physiological significance? The latter question relies for an answer on a knowledge of the intracellular concentrations of both PEP and FDP, and the estimations of the concentrations of these intermediates unfortunately leave much to be desired. The calculations carried out at the beginning of this chapter indicated that as assayed in the standard system at 1.67×10^{-3} M-PEP, the amount of enzyme present is not adequate to account for the total utilisation rate of PEP *in vivo*. When estimations of the PEP content of whole cells were carried out, in the following manner, the same conclusion was reached.

Trypanosomes were suspended in dilute solution (1 ml packed cell volume/40 ml saline) with an excess of glucose (1000 µmoles) and maintained at room temperature for ten minutes. The cells were rapidly sedimented at 3000g and $2^{\circ}C$ and the supernatant withdrawn and discarded. Perchloric acid (0.33 M) was added to the trypanosome pellet and the

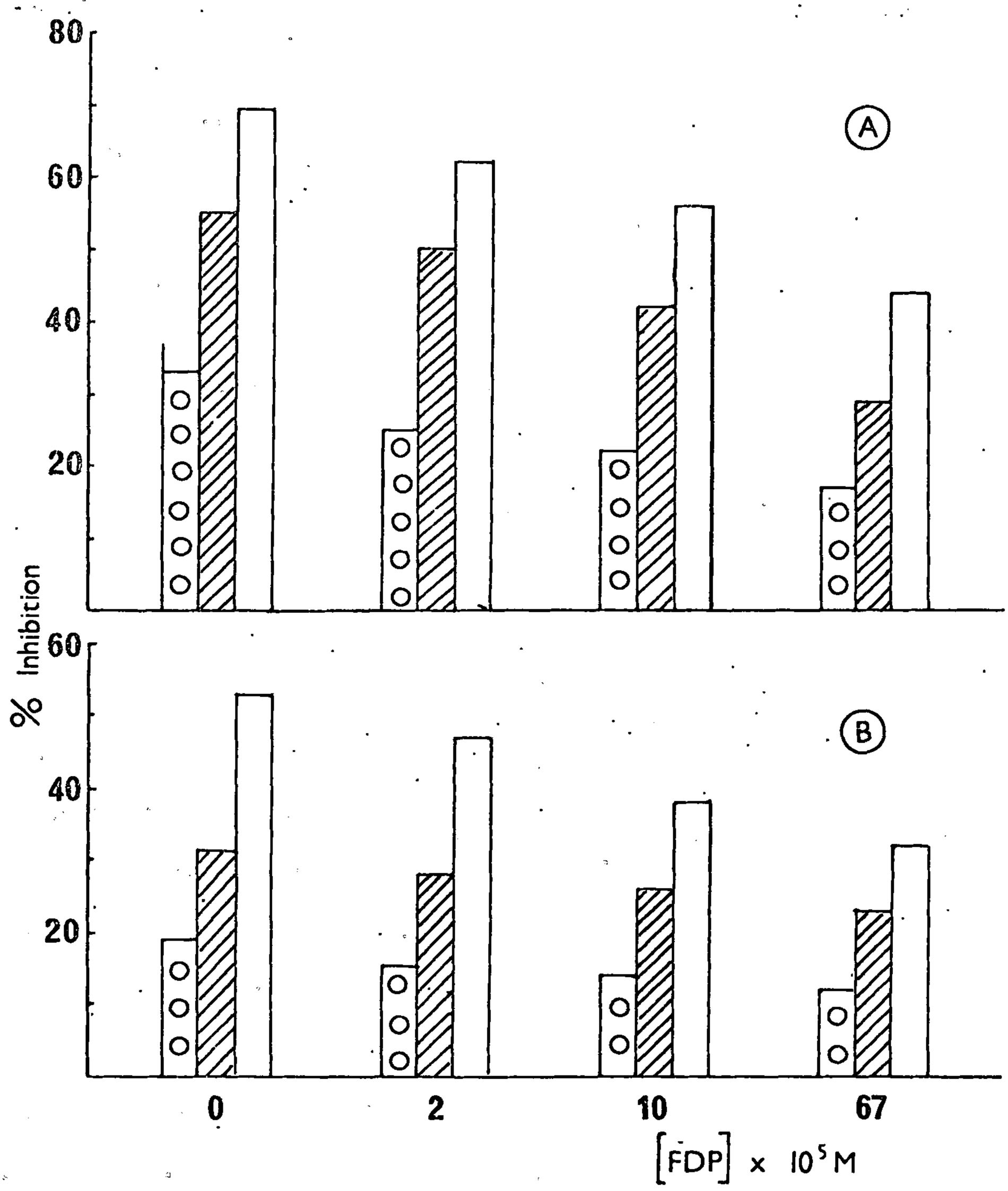


Fig. 40. The effect of FDP on the melarsen oxide inhibition of purified pyruvate kinase. Aliquots of enzyme, after the removal of DTT, were preincubated in the standard assay medium plus 1.00 mM-EDTA for 5 minutes with and without melarsen oxide and FDP, prior to initiation of the reactions by addition of ADP (4.17×10^{-4} M) and PEP (A: 0.67 mM. B: 1.67 mM). The concentration of melarsen oxide was as follows.
[O] = 10^{-5} M. [▨] = 3×10^{-5} M. [ ] = 10^{-4} M.

material homogenised. After neutralisation with dipotassium hydrogen phosphate (0.5 M), PEP was estimated in the supernatant, with the following results:

	Cell volume	PEP present	"Intracellular concentration"
1)	0.52 ml	0.19 µmole	3.7×10^{-4} M-PEP
2)	0.91 ml	0.42 µmole	4.6×10^{-4} M-PEP
3)	0.41 ml	0.21 µmole	5.1×10^{-4} M-PEP

It is realised that these figures can give only the roughest indication of the true value. Firstly, sedimentation of the organisms takes a finite time, and they are in the "packed" state for approximately three minutes prior to lysis with the perchloric acid. Under these conditions, every organism is in an artificial micro-environment of rapidly decreasing glucose concentration and even more rapidly increasing pyruvate concentration, and the parasites become exceedingly sluggish in their movements. Hence the build-up of pyruvate conceivably will increase the PEP concentration by product inhibition of the pyruvate kinase. Secondly, the "packed cell volume" gives only the vaguest indication of the total cell volume present, and no indication at all of the percentage of the intracellular volume available to the enzymic substrate. However, the figures as they stand substantiate the idea that *in vivo*, the enzyme requires FDP activation to function at the calculated rate.

It was therefore necessary to investigate the mode of action of melarsen oxide on the enzyme in the presence of FDP. Figure 40 demonstrates the apparent competition between melarsen oxide and FDP, in so far as the drug I_{50} value increases with increasing FDP concentration. This protection of the enzyme by FDP is independent of the mode of action of the metabolic activator, i.e. a progressive effect of FDP is found through the concentration range at which the PEP Hill

TABLE 23

Effect of FDP on the kinetic parameters of normal and melarsen oxide inhibited purified pyruvate kinase

Experimental details as in Figure 39, with varying concentrations of FDP present during the preincubation period. Parameters calculated as previously described and quoted ± SD. Melarsen oxide concentration = 10^{-4}M.

Concentration of FDP (M)	S_{50} PEP (mM)		n_{PEP}		$\frac{V_{max}}{(\mu mol/min/ml\ enzyme)}$	
	Standard	+ Melarsen oxide	Standard	+ Melarsen oxide	Standard	+ Melarsen oxide
Zero	1.81 ± 0.12	3.30 ± 0.09	1.89 ± 0.04	1.92 ± 0.03	1.31 ± 0.06	1.29 ± 0.04
2.0×10^{-5}	1.62 ± 0.04	3.47 ± 0.07	1.41 ± 0.03	1.52 ± 0.03	1.24 ± 0.04	1.29 ± 0.05
6.7×10^{-4}	0.46 ± 0.06	1.29 ± 0.12	0.99 ± 0.04	1.02 ± 0.04	1.32 ± 0.07	1.16 ± 0.05

coefficient is the primarily affected parameter, and further protection is found in the region of FDP concentration at which the PEP S_{50} value is the variable (c.f. Figure 34).

Stimulation of the enzyme by FDP, however, has no effect on the interaction between PEP and melarsen oxide (Table 23). An elevated S_{50} PEP value is found in the presence of melarsen oxide over the range of FDP concentrations from zero to 6.7×10^{-4} M. Over the same range of activator concentrations, the presence of the drug produces no change in either the Hill coefficient for PEP, or the maximum velocity. Thus an identical picture is presented in the presence and in the absence of FDP, in which the drug is competing with the enzyme substrate.

Effects of other inhibitors

The process of enzyme purification, as far as may be judged, did not drastically modify the properties of the kinase. The K_M value for $MgADP^-$ decreases from 12×10^{-5} M to 8×10^{-5} M-ADP, and the I_{50} value for melarsen oxide similarly drops from 10^{-4} M to 4.3×10^{-5} M when freshly purified enzyme is used. The pattern of inhibition exhibited by the purified material is very similar to that of the crude enzyme, the effects of various compounds on the purified enzyme being defined in Table 24 (c.f. Table 19).

The sensitivity of the pure enzyme to these inhibitors is, with the exception of pCMB, uniformly greater than that of the unfractionated enzyme. Inorganic arsenite, iodoacetate and iodoacetamide, however, still do not inhibit the system. Of the inhibitors tested, pCMB shows the greatest inhibitory action, with an I_{50} value approximately 100-fold lower than those of the arsenical derivatives. The parent drug, phenylarsenoxide, and its p-amino- derivative (reduced atoxyl) interact

TABLE 24

Effect of inhibitors on purified pyruvate kinase

Enzymic rates were measured after five minutes preincubation in the presence or absence of inhibitors. Each I_{50} value is from at least five inhibitor concentrations and the figures quoted are the mean values of the number of determinations shown in parentheses. Competitive inhibition implies an increased S_{50} PEP value with an unchanged V_{max} value. Non-competitive inhibition implies an unchanged S_{50}PEP with a decreased V_{max}. The concentration of PEP was maintained at 1.67 mM for the determination of ID_{50} values.

Inhibitor	ID_{50} (M)	Mode of inhibition with respect to PEP
Phenylarsenoxide	1.9×10^{-4} (3)	Competitive
p-aminophenylarsenoxide	7.9×10^{-4} (2)	Competitive
Sodium arsenite	N.I. (4)	-
pCMB	9.2×10^{-7} (2)	Non-competitive
Iodoacetate	N.I. (1)	-
Iodoacetamide	N.I. (1)	-
N-ethyl maleimide	2.6×10^{-4} (2)	*
Melamine	N.I. (1)	-

N.I. denotes not inhibitory at the inhibitor concentrations quoted in Table 19.

* denotes not determined.

with the enzyme in the same fashion as the melaminyl drug, purely competitive kinetics being obtained. On the other hand, the inhibition by pCMB gave a decrease in the maximum velocity, with no change in the S_{50} value and Hill coefficient for PEP.

8) MAMMALIAN PYRUVATE KINASES

During the early part of the study of the trypanosome PK, the commercially available rabbit muscle enzyme was conveniently used to test the validity of the assay system, and as a comparative host enzyme. However, the observations on the allosteric nature of the trypanosome enzyme as regards the cooperative effects of PEP and the action of FDP, make this comparison to a certain extent artificial, as the muscle enzyme is allosterically inactive in these respects. The kinetic properties of the trypanosome enzyme are much more similar to those of the allosterically controlled liver L isoenzyme. The presence in mammalian liver of both L and M forms of PK offered the opportunity of testing the hypothesis that arsenical sensitivity in pyruvate kinase is correlated with the presence of allosteric control of the enzyme. The L isoenzyme, akin to the parasite enzyme, was expected to be arsenical sensitive. The non-allosteric M isoenzyme, by comparison with the rabbit muscle system should have been insensitive to the trypanocide. This hypothesis was not, however, entirely borne out, as may be seen from the work reported below.

Preparation of liver isoenzymes.

The method used to separate the isoenzymes from rat liver is an adaptation of the method of Passeron *et al.*, (1967). The L-isoenzyme of rat liver is highly unstable, another point of similarity to the parasite enzyme, and the characteristics of both L and M type proteins

change on storage. To avoid complication of the kinetic results in this way, freshly prepared enzyme samples (< 24 hours old) were used unless otherwise stated. The use of fresh material daily also allowed mercaptoethanol, recommended by Passeron as a stabilising agent, to be eliminated from the enzyme solution during the purification. In this way no interference with the arsenical reaction by exogenous thiol is possible.

The livers of two rats were excised, weighed, and homogenised in 1 volume of cold 0.1 M-tris buffer, pH 7.5, containing EDTA (10^{-3} M), and sucrose (25% ($^{W}/v$)). (The high protein concentration used here increases the yield of the delicate L isoenzyme in the absence of mercaptoethanol). The tissue homogenate was centrifuged for 1 hour at 60,000g and the supernatant removed for further fractionation. To this supernatant was added the above mentioned Tris/sucrose/EDTA buffer, saturated with ammonium sulphate, to give a final concentration of 20% saturation with respect to the ammonium salt. After standing in ice for 30 minutes, the sediment was collected by centrifugation at 10,000g for 30 minutes and was discarded. The supernatant was brought to 45% saturation in ammonium sulphate, and after a further 30 minutes, the sediment was collected as above. This sediment was redissolved in a minimal volume of tris/EDTA/sucrose, and passed through a G-25 Sephadex solumn equilibrated with the same solution, the protein containing fractions being [combined], and stored. The supernatant from the 45% saturated ammonium sulphate solution was brought to 55% saturation, the sediment removed as above and discarded, and the 55% saturated supernatant made up to 70% saturation as before. The resulting 55% - 70% sediment was redissolved and desalted as for the 20-45% fraction. According to Tanaka (1967a,b) this procedure should ensure separation of

the isoenzymes, the L type precipitating at 20-45% saturation in ammonium sulphate, the M type in the higher range of concentrations. However, to ensure as far as is possible that total separations were obtained, the individual desalted fractions were applied to a column of DEAE cellulose, pre-equilibrated in 0.05 M-tris pH 7.5/10^{-3} M-EDTA, and eluted with a KCl gradient. The L isoenzyme, already concentrated in the ammonium sulphate fraction (20-45% saturation) was eluted at 0.02 - 0.04 M-KCl whereas the M isoenzyme is released from the column at 0.12 - 0.14 M-KCl.

Purity and stability of the liver isoenzymes

Neither L nor M pyruvate kinase is produced in a completely pure state by the procedure outlined above, and in fact the latter has been shown to contain low concentrations of enolase ($\leq 3\%$ PK activity). However, as far as may be ascertained, they are free from cross-contamination by the other isoenzyme in the final state. The elution profiles from DEAE-cellulose showed that after the ammonium sulphate separation, the L isoenzyme contained approximately 4% of the M type protein, and the selected M type fraction, about 12% of L type enzyme. Reapplication of the DEAE-cellulose purified fractions to a second column of the same material, or a second ammonium sulphate fractionation, showed the separated isoenzymes to be homogeneous with respect to the type of PK present. The kinetic pictures obtained with these semi-purified fractions lend strength to their mutually exclusive purity, and no further purification was undertaken.

The crude, as well as the purified L type enzyme was found to be highly susceptible to inactivation under a variety of circumstances, and as the kinetic characteristics of both isoenzymes were mutable on storage (see below), the data presented are from experiments on fresh preparations

(i.e. < 24 hours after purification) unless otherwise stated.

Kinetic properties of the liver isoenzymes.

From the work presented earlier in this thesis, the hypothesis that inhibition by melarsen oxide is connected with the allosteric properties of the enzyme, accounting for the absence of interaction between the drug and the muscle enzyme, was presented. Preliminary experiments showed, however, that contrary to the expected result, both liver isoenzymes were susceptible to inhibition by the arsenical. A closer investigation of the kinetic properties of the enzymes involved was therefore carried out.

Interaction with ADP

Both isoenzymes appear to be totally dependent on both a monovalent and a divalent cation for activity: To avoid difficulties in interpretation, the assay system used was identical with that used for the parasite enzyme.

With both enzymes hyperbolic responses were obtained when the concentration of the nucleotide coenzyme was varied, and again in both cases, the K_M values obtained were independent of the concentration of PEP. The K_M (apparent) values obtained were;

L- isoenzyme 2.61×10^{-4} M-ADP.

M- isoenzyme 2.32×10^{-4} M-ADP.

Interaction with PEP.

i. L type isoenzyme.

Freshly prepared specimens of this protein showed a marked homotropic interaction between PEP molecules, and the mean kinetic values obtained from four such samples are as follows.

S_{50} value: $2.18 \pm 0.41 \times 10^{-4}$ M_o-PEP

Hill coefficient: 1.81 ± 0.12.

Storage of the enzyme resulted in a marked decrease in the allosteric properties, and after four days at $+2^{\circ}C$, the mean Hill coefficient value decreased to 1.32 ± 0.14. If the enzyme is stored at room temperature (in the presence of thymol as a bacteriostat) less desensitisation occurs, and after four days the Hill coefficient was = 1.64 ± 0.10.

ii. M type isoenzyme.

Kinetic investigation of this isoenzyme showed clearly that the original statement of Tanaka et al., (1965) that "Pyruvate kinase M is an usual muscle type enzyme" is untrue. Fresh preparations gave values for an interaction coefficient between PEP molecules of between 1.40 and 1.62 but after storage, either at room temperature or in the cold, this value rapidly decreased to 1.02 - 1.22. Later work reported by these authors agrees with this finding, as they quote a value for n of 1.4 for this isoenzyme, thus clearly differentiating it from the muscle protein (Tanaka et al., 1970).

Interaction with FDP.

In both isoenzyme systems, the response to PEP concentration is transformed in the presence of this activator (10^{-3} M) to give a hyperbolic curve, and the affinity of the enzymes for the substrate is enhanced.

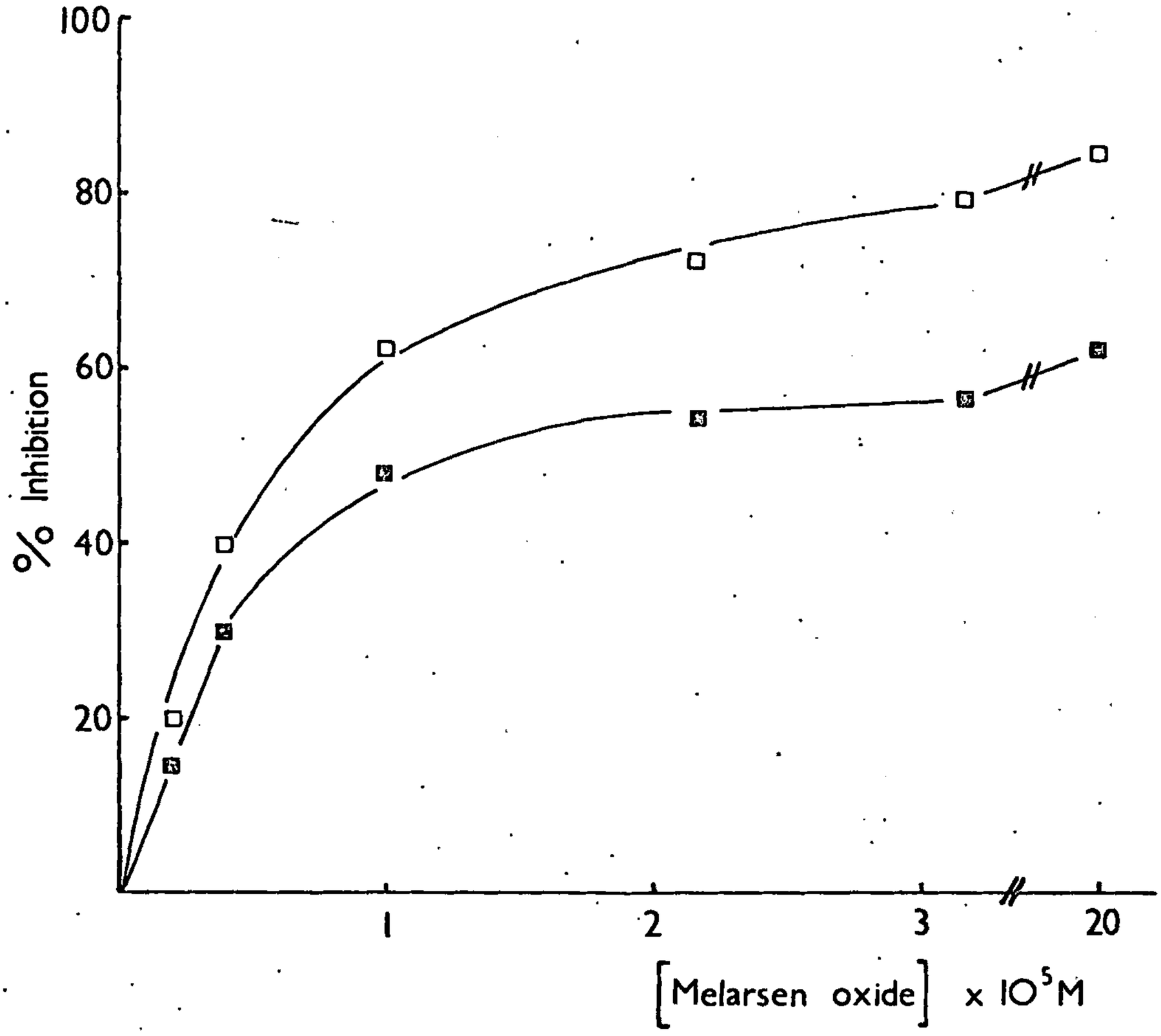


Fig. 41. The effect of melarsen oxide on the isoenzymes of liver pyruvate kinase. Isoenzymes were separated as described in the text. Aliquots (approx. 0.1 I.U.) were preincubated for 5 minutes (L isoenzyme, □) or 30 minutes (M isoenzyme, ■) in the standard assay medium plus 1.0 mM-EDTA, with and without melarsen oxide. Reactions were initiated by addition of ADP (4.17×10^{-4}M) and PEP (1.67 mM). Both enzyme fractions were less than four hours old.

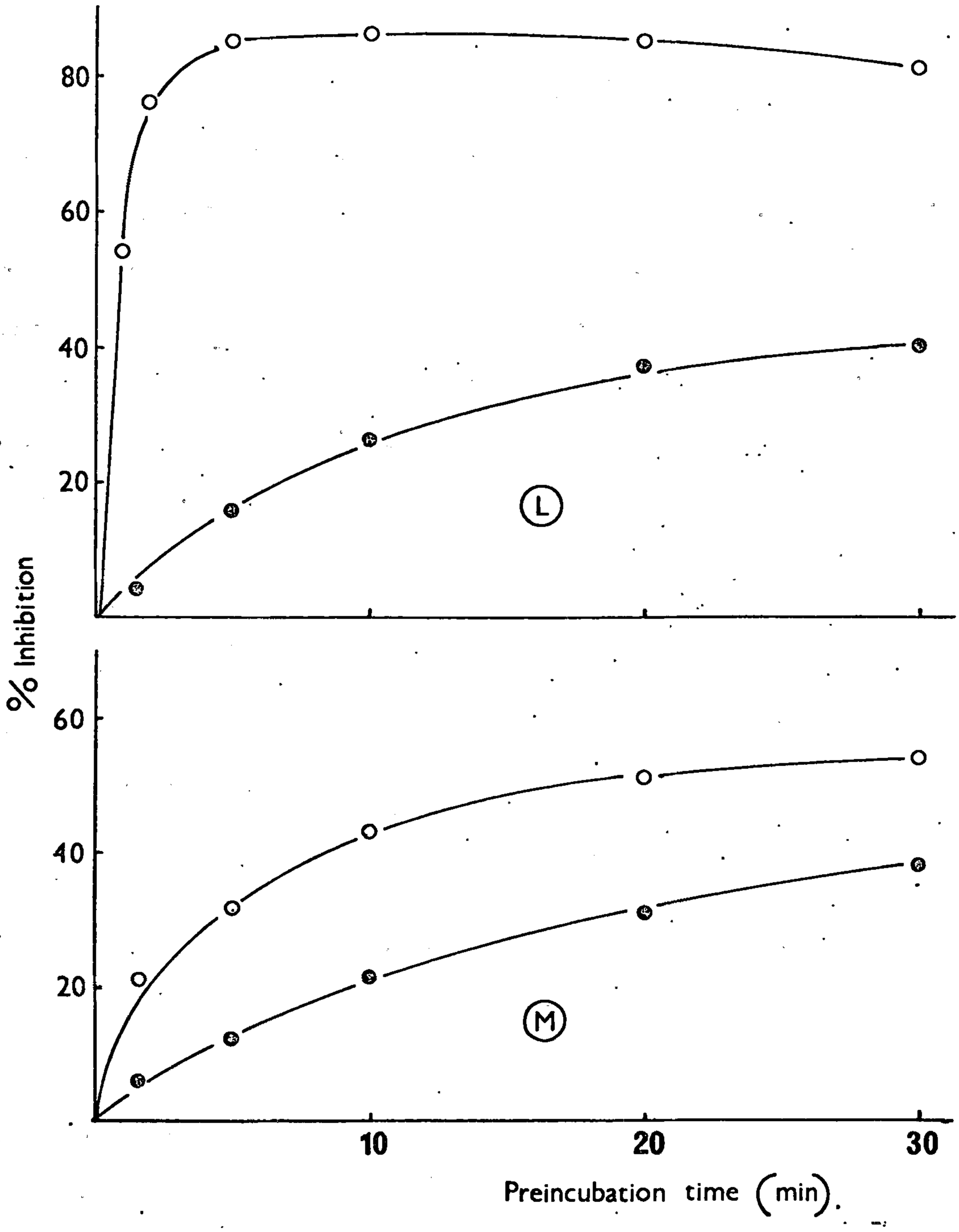


Fig. 42. The effect of preincubation time on the inhibition by melarsen oxide of the isoenzymes of liver pyruvate kinase. All experimental assay conditions as described in Fig. 41, with a concentration of melarsen oxide of 3×10^{-5} M. O = freshly prepared enzyme. ⊛ = enzyme after storage for 3 days at 2°C. Isoenzymes as indicated on the diagram.

Apparently, then, both forms of the enzyme are heterotropically activated as well as being subject to homotropic substrate activation.

Interaction with melarsen oxide.

Storage of the two liver enzymes led to desensitisation to the effect of melarsen oxide as well as to the allosteric interaction of PEP binding sites. Also, despite the presence of EDTA in the assay medium, the reactions between enzymes and melarsen oxide were exceptionally time-dependent.

Under the conditions used for assay of the trypanosome enzyme, the responses to variation in the drug level are illustrated in Figure 41. The L type enzyme appears to be rather more sensitive to the drug, with an I_{50} value under these assay conditions of 6×10^{-6} M-melarsen oxide as opposed to the value of 1.2×10^{-5} M-melarsen oxide obtained for the M type enzyme. The Hill coefficients obtained at the time for these samples of enzyme were L : 1.72 M : 1.43.

On the following diagram (Figure 42) may be seen the combined effects of preincubation time and length of storage (at $+2^{\circ}C$) on the two isoenzymes. Both forms are subject to desensitisation on storage, and further, the time of incubation required to attain equilibrium is dependent upon the duration of storage. (It should be noted that in the experiments described by Figure 41 the preincubation periods for the respective enzymes are those at which inhibition appears to be complete).

Despite the support lent to the above-mentioned bypothesis by the parallel desensitisation to allosteric activation and to drug induced inhibition, no further work was carried out on the stored enzymes. However, the relatively enormous (compared to the trypanosome enzyme) quantities of these enzymes which were available, did enable studies to

TABLE 25

Effect of preincubation time on the kinetic parameters of PEP utilisation by liver pyruvate kinases

Enzyme (approx. 0.05 IU) was incubated in the standard assay medium in the presence or absence of melarsen oxide (8×10^{-6}M) for the preincubation times stated, prior to initiation of the reactions by addition of ADP (3 µmoles) and PEP. Each set of parameters is from at least eight concentrations of PEP from 2×10^{-5} M-PEP to 5×10^{-3}M-PEP. Preincubation was at room temperature.

		S_{50} ($\times 10^4$M)		V_{max} (µmol/min/mg protein)		n_{PEP}		
Enzyme	Preincubation time (min)	Standard	+ Melarsen oxide	Standard	+ Melarsen oxide	Standard	+ Melarsen oxide	Type of inhibition
M	5	1.27	9.47	10.6	10.4	1.39	1.41	Competitive
M	5	1.41	8.62	8.3	8.6	1.26	1.21	Competitive
M	35	5.32	5.64	7.7	3.6	1.21	1.22	Non-competitive
M	40	5.41	5.12	8.1	3.4	1.18	1.26	Non-competitive
L	5	2.18	7.31	18.4	19.1	1.81	1.82	Competitive
L	8	2.39	6.07	14.3	14.1	1.69	1.74	Competitive
L	10	2.61	5.82	12.6	9.8	1.73	1.68	? Mixed
L	35	4.81	4.96	8.2	4.4	1.84	1.69	Non-competitive
L	40	5.22	4.92	8.6	5.1	1.61	1.80	Non-competitive
L	40	4.73	5.21	10.4	6.3	1.62	1.80	Non-competitive

be carried out on the effects of incubation time on the mode of inhibition by the arsenical.

Both isoenzymes portrayed the same pattern of inhibition, as follows. When a short preincubation time was used (< 10 minutes), melarsen oxide appeared to be a competitive inhibitor with respect to PEP, although in one freshly purified sample of the L type enzyme, mixed inhibition was found, where the S_{50} PEP value was increased and the V_{max} was decreased. If the length of the preincubation period was increased to 40 minutes, non-competitive kinetics were obtained on variation of the PEP concentration. The S_{50} PEP values and V_{max} values for these experiments are shown in Table 25.

Other mammalian pyruvate kinases.

Preliminary investigation of some other sources of pyruvate kinase has been undertaken, on Sephadex G-25 treated homogenates of certain organs as follows. Rat heart and brain were individually homogenised in 0.05 M-Tris pH 7.5 / 10^{-3} M-EDTA and the supernatant from a 10,000g centrifugation for 1 hour desalted as previously described for the liver isoenzymes. The pyruvate kinase from these sources had Hill coefficients for PEP of 1.02 $\pm$ 0.04 (brain) and 0.96 $\pm$ 0.07 (heart) and neither enzyme was inhibited by 10^{-4} M-melarsen oxide after 15 minutes preincubation with the drug in the previously described fashion. Another property shared by these enzymes and the commercial rabbit muscle enzyme, is their insensitivity to FDP up to 10^{-3} M.

A recent report (Rozengurt et al.,) 1970 has shown that the enzyme from mammalian muscle is not totally allosterically inactive, being inhibited by phenylalanine. Fresh extracts of rat muscle, however, when

prepared in the same fashion as the heart and brain enzymes, showed no cooperativity between PEP molecules ($n = 0.98 \pm 0.02$), no activation by 10^{-3} M-FDP, and no inhibition by melarsen oxide (10^{-4} M). The lack of response of the commercial enzyme thus does not appear to be an artefact induced by prolonged refrigerated storage.

CHAPTER 6

DISCUSSION

1) CARBOHYDRATE METABOLISM OF PLEOMORPHIC T.RHODESIENSE

The intense motility of the bloodstream forms of T.rhodesiense is supported by an extremely high rate of utilisation of extracellular glucose, corresponding to at least 50% of the dry weight of the organisms per hour. This absolute requirement for a source of energy is common to both monomorphic and pleomorphic strains, and in the absence of such a source the organisms rapidly become immotile and degenerate. With a monomorphic strain, the results presented here would indicate, in common with the work of Ryley (1956) and Grant and Fulton (1957), that the glucose is practically entirely degraded to pyruvate, with a negligible production of respiratory carbon dioxide.

Oxidative decarboxylation

There are two major differences between freshly isolated pleomorphic, and old laboratory maintained monomorphic trypanosomes. Firstly, the pleomorphs contain active oxidative decarboxylation systems for the catabolism of pyruvate and α-OG, and secondly, significant quantities of metabolic carbon dioxide are produced during the utilisation of glucose or glycerol. The pyruvate and α-OG decarboxylases appear to be confined to the short stumpy bloodstream forms (Figure 6). These results are in agreement with the observation of Vickerman (1965), that α-OG preferentially maintained the motility of the stumpy forms. Whereas the stumpy forms utilise extracellular α-OG efficiently and remain motile in this substrate, exogenous pyruvate will not maintain the organisms. This implies a specificity in the properties of the trypanosomal membranes, as the CO_2 production from glycerol utilisation indicates the decarboxylation of endogenously synthesised pyruvate.

Removal of endogenous cofactors from cell lysates produced low yields of these relatively unstable oxidative decarboxylases. However, it was possible to show that coenzyme A, NAD^+ and ADP significantly stimulated these enzymes (Table 5). Attempts to reconstitute an active tricarboxylic acid cycle in cofactor-depleted preparations, by the addition of aspartate or malate as sources of oxaloacetate, did not stimulate the utilisation of pyruvate, which appears to be utilised by a one-step oxidative decarboxylation reaction in which acetyl coenzyme A is synthesised and subsequently hydrolysed to produce acetate. Similarly, α-OG is decarboxylated to succinate, the limiting activity of succinate dehydrogenase prohibiting further utilisation (Table 9).

Oxygen utilisation

Oxygen utilisation by pleomorphic strains of trypanosomes with glucose as substrate resembles that of the syringe-passaged strains in being insensitive to cyanide, and, consistent with this observation, they contain no detectable amounts of cytochrome pigments. The differential effects of cyanide on whole cells and lysates utilising α-OG are puzzling. It is improbable that a cyanide-sensitive pathway for oxygen utilisation is available to this substrate alone in the lysed preparation, but not in the whole organism. Possibly the cyanhydrin which is known to be formed between α-OG and cyanide may act as a competitive inhibitor of the α-oxoglutarate oxidase system. The lack of effect *in vivo* may then be due to the impermeability of the cell membrane to cyanide, to the cyanhydrin, or to both.

In the absence of a functional cytochrome chain, the substrate-independent inhibitory effects of rotenone and amytal are not expected. The lack of effect on α-glycerophosphate utilisation in the lysed

preparations eliminates the α-glycerophosphate oxidase as the focal point of these inhibitors. However, this substrate differs from glucose, α-OG and pyruvate, in being directly linked to the oxygen utilising enzyme, without the intervention of NAD^+ as a coenzyme.

Polarographic investigation of NADH reoxidation showed the presence of a DHAP-independent pathway as well as the DHAP dependent α-glycerophosphate oxidase of Grant and Sargent (1960). The rate of NADH oxidation is markedly stimulated by addition of fructose 1;6 diphosphate as a source of DHAP, and both DHAP-dependent and independent pathways to oxygen are rotenone sensitive. These results, taken with the quantitatively similar I_{50} values of rotenone and amytal on glucose, α-OG and pyruvate metabolism, point to α-glycerophosphate dehydrogenase, catalysing the reduction of DHAP to L-α-GP by NADH, as the sensitive enzyme. Spectrophotometric assay of this enzyme, however, did not bear out this hypothesis. On the other hand, the sensitivity of the monomorphic strain to these inhibitors, argues against the effect being mediated elsewhere than at the α-glycerophosphate dehydrogenase step.

These data may be summarised as follows:

Method of estimation		Substrate	Rotenone sensitivity
Polarographic	1	NADH	+
	2	NADH + FDP	+
	3	L-α-GP	-
Spectrophotometric	1	NADH	+
	2	NADH + FDP	-

The apparent anomaly in these data cannot at present be satisfactorily accounted for, and this system deserves further investigation.

However, it appears that the pleomorphic strains overcome the problem of reoxidation of metabolic NADH in a similar fashion to the monomorphic strains, being largely dependent upon the L-∝-glycero-phosphate oxidase system.

End products and routes of glucose metabolism.

In the first instance, the formation of significant amounts of CO_2 from glucose metabolism accompanied by a marked decrease in the pyruvate yield, suggested the possibility of a functional tricarboxylic acid cycle in SS forms, despite the lack of a cytochrome chain. Clarification of this possibility has been attempted by a study and balance of the end-products of glucose catabolism.

Further metabolism of glycolytic pyruvate is confined to an oxidative decarboxylation reaction producing acetate (Table 11). Exogenous pyruvate is only utilisable by lysed cell preparations and its metabolism appears to involve the production of a coenzyme A derivative as an intermediate (Table 5). Citrate synthase is present in very low concentration in T.rhodesiense and the inadequacy of this enzyme would account for the failure of malate, aspartate or oxaloacetate to stimulate citrate synthesis from pyruvate.

The other tricarboxylic acid cycle enzyme which is present in exceedingly low activity in this organism is succinoxidase. Thus ∝-oxoglutarate is metabolised only as far as succinate by lysed or whole cells, although a small proportion (< 2%) of the succinate may be further metabolised to oxaloacetate and thence decarboxylated to produce pyruvate (Table 11).

Aerobic glucose metabolism results in the formation of acetate, presumably via pyruvate, and also of significant quantities of glycerol.

TABLE 26

A comparison of the observed and theoretical gas exchanges of T.rhodesiense EATRO 173 utilising glucose.

The metabolite formation data of Table 11 were used to compute the theoretical gas exchanges for pathways 1 - 4 as described in the adjacent text.

Experimental duration (mins)	Observed data			Pathway 1			Pathways 2 & 4			Pathway 3		
	O_2 utilised (µmoles)	CO_2 produced (µmoles)	R.Q.	O_2 utilised (µmoles)	CO_2 produced (µmoles)	R.Q.	O_2 utilised (µmoles)	CO_2 produced (µmoles)	R.Q.	O_2 utilised (µmoles)	CO_2 produced (µmoles)	R.Q.
10	2.88	0.72	0.25	2.38	1.04	0.44	2.38	0.90	0.38	2.03	0.48	0.24
10	2.31	0.46	0.20	2.04	0.78	0.38	2.04	0.72	0.35	1.89	0.54	0.29
20	3.73	1.14	0.31	3.35	1.23	0.37	3.35	1.04	0.31	2.88	0.66	0.23
20	4.59	1.18	0.26	3.66	1.67	0.46	3.66	1.43	0.39	3.06	0.71	0.23
30	5.76	2.42	0.42	4.89	2.66	0.54	4.89	2.21	0.45	3.76	0.85	0.23
30	4.72	1.48	0.31	4.28	1.93	0.45	4.28	1.62	0.39	3.73	1.00	0.27
45	8.02	4.04	0.50	7.56	4.35	0.58	7.56	3.57	0.47	5.62	1.23	0.22
45	8.56	3.96	0.46	7.87	3.48	0.44	7.87	2.98	0.38	6.61	1.48	0.22
50	8.35	4.34	0.52	8.43	4.38	0.52	8.43	3.66	0.43	6.20	1.50	0.24
50	8.85	3.51	0.40	7.61	4.26	0.56	7.61	3.51	0.46	5.73	1.25	0.22
50	7.05	2.78	0.35	6.62	3.22	0.49	6.62	2.51	0.38	5.85	1.09	0.19
60	8.38	4.64	0.55	7.95	5.06	0.64	7.95	4.10	0.52	5.55	1.22	0.22
60	8.86	5.35	0.60	8.71	5.29	0.61	8.71	4.39	0.50	6.46	1.69	0.26
60	10.10	4.62	0.46	9.17	6.07	0.66	9.17	4.85	0.53	6.73	1.19	0.18
70	9.52	4.93	0.52	9.92	5.37	0.54	9.92	4.10	0.41	6.74	1.56	0.23

Glycerol is accumulated by both monomorphic and pleomorphic strains, and will enable the redox balance of the organism to be maintained without utilisation of oxygen. It is worthy of note that the culture form of T.rhodesiense does not accumulate glycerol during either aerobic or anaerobic metabolism of glucose (Ryley, 1962).

The production routes of CO_2 and succinate are not so readily defined. The evolution of CO_2 is greater than that involved in the formation of acetate and hence must come partially from an alternative source. It is possible to estimate the theoretical gas exchange expected from the production of a measured amount of a metabolite via various metabolic pathways, and a comparison of the theoretical and experimental exchanges may clarify the actual route involved. The mechanism of succinate formation has been examined in this manner with the data from Table 11, and the carbon-balance data are re-presented in Table 26. The theoretical gas exchanges shown are for four possible modes of succinate production. The formation of pyruvate is assumed to occur glycolytically, yielding the equivalent reducing power for 1 mol O_2 per 2 mols pyruvate produced. Acetate is assumed to come from the decarboxylation of pyruvate with an R.Q. of 2.0, and glycerol from the dephosphorylation of L-α-glycerophosphate. This last process involves the reduction of glycolytic DHAP by NADH, and hence the glycerol formed will detract from the theoretical uptake of oxygen in the formation of pyruvate from glucose.

Pathway (1): Pyruvate is further metabolised by the tricarboxylic acid cycle, terminating at succinate. Oxaloacetate required for this process is assumed to come from a source other than the direct products of glucose catabolism.

Pathway (2): As for pathway (1), only the oxaloacetate is assumed to be produced by CO_2 fixation involving PEP or pyruvate.

Pathway (3): Assumes that a CO_2 fixation step onto PEP or pyruvate is followed by reduction of the product to succinate. The tricarboxylic acid cycle is assumed to be inoperative.

Pathway (4): The source of succinate is via the glyoxalate cycle which simultaneously regenerates oxaloacetate.

The oxygen consumption in all but two experiments is greater than any of the theoretical uptakes. This may reflect the metabolism of the 4-12% of the utilised glucose which is not accounted for in the balances. The yields of CO_2 may best be described as a combination of pathways (3) and (2 or 4) at experimental periods of up to 30 minutes, and as a combination of pathways (1) and (2 or 4) at longer experimental periods. It has been shown that changes in the metabolic pattern occur during an incubation period of one hour, and it is possible that not only the relative yields of metabolites change during incubation, but also the metabolic paths. Complete definition of the pathways involved is not possible, due to the metabolism of the glucose which is not accounted for in these balances. Complete oxidation of this substrate would disrupt the balances, and large variation from the theoretical exchanges would be expected. This problem can only be resolved by means of a ^{14}C balance to determine the origin of the succinate carbons directly.

Over the time course of the incubations, yields of glycerol, succinate, and CO_2 per glucose utilised progressively increase with the time of incubation, and pyruvate production and O_2 utilisation per glucose utilised decrease. The formation of acetate remains constant in the range 0.25 - 0.30 μmole / μmole glucose utilised (Figure 7a-d). The increase in the CO_2 yield cannot therefore be related to an increase in the decarboxylation of pyruvate to yield acetate. The apparent absence of 6-phosphogluconate dehydrogenase in these organisms makes the Lipmann-Dickens

shunt an unlikely source of this CO_2, and no CO_2 is evolved in the formation of glycerol. Thus, the increase in CO_2 production is most probably associated with the increase in succinate production via the tricarboxylic acid or glyoxalate cycles. The CO_2-fixation routes would obviously decrease the yield of CO_2. The decrease in oxygen utilisation may be correlated with the increase in glycerol formation, thus regenerating NAD^+ anaercbically.

This time-dependence of the metabolite yields may reflect a morphological deterioration of the organisms, or some chemical effect. The former is unlikely as the organisms appear microscopically normal on inspection after incubation. In vitro, however, a large pool of pyruvate accumulates which in vivo would be removed via the plasma. This large increase in pyruvate formation may have the effect of instigating or exaggerating the use of pathways not normally acting at a significant rate. The experimental organisms most closely approximate to their in vivo state at the time at which very little pyruvate has accumulated. This will be at the start of the preincubation period, at a point -10 minutes on Figures 7a - d. These data have been extrapolated to this point by the fitting of unweighted regression lines with the following results:

Product	µmol / µmol glucose ± S.D. at -10 mins.		% Glucose
Pyruvate	1.507 ± .041		75
Succinate	.021 ± .009		1
CO_2	.199 ± .046		3
Acetate	.277 ± .020		9
Glycercl	.096 ± .019		5
		TOTAL	93%
Oxygen utilisation	1.278 ± .012		
R.Q.	.124 ± .042		

It would appear then that the high yields of succinate and CO_2 obtained during prolonged incubation may be artefacts of the incubation conditions, rather than the consequences of the normal metabolism of the organism. The R.Q. is very low, and approaches that previously reported for a monomorphic strain (Ryley, 1956; 1962). Within the limits of error, all the CO_2 production can now be accounted for on the basis of acetate formation. However the utilisation of oxygen remains anomalously high, as the maximum uptake on the basis of the pyruvate / glycerol / succinate figures is 1.024 µmol / µmol glucose, by pathways (1) (2) or (4). The excess oxygen may be utilised in the metabolism of the remaining 7% of the glucose, any production of CO_2 being used possibly by fixation to form oxaloacetate.

This discussion has been based on the simplifying assumption that all the organisms in a trypanosome sample are metabolically equivalent. The semi-quantitative aspects would not be changed, however, if the trypanosome population consisted of a mixture of metabolic types, some of which relied purely on glycolysis and produced 100% pyruvate from glucose, and others which totally metabolised the substrate to CO_2 and H_2). The dependence of the α-oxoglutarate and pyruvate utilisation rates on the % of SS forms in the samples (Figure 6) would indicate that at least two metabolically different organisms are present in the infections.

Summary.

The pleomorphic infection of T.rhodesiense appears to rely on the same terminal oxidase system as the passaged monomorphic strains to maintain its aerobic redox balance. In such pleomorphic strains, two biochemically different organisms exist with regard to their competence in the catabolism of glucose. This difference can be traced to the

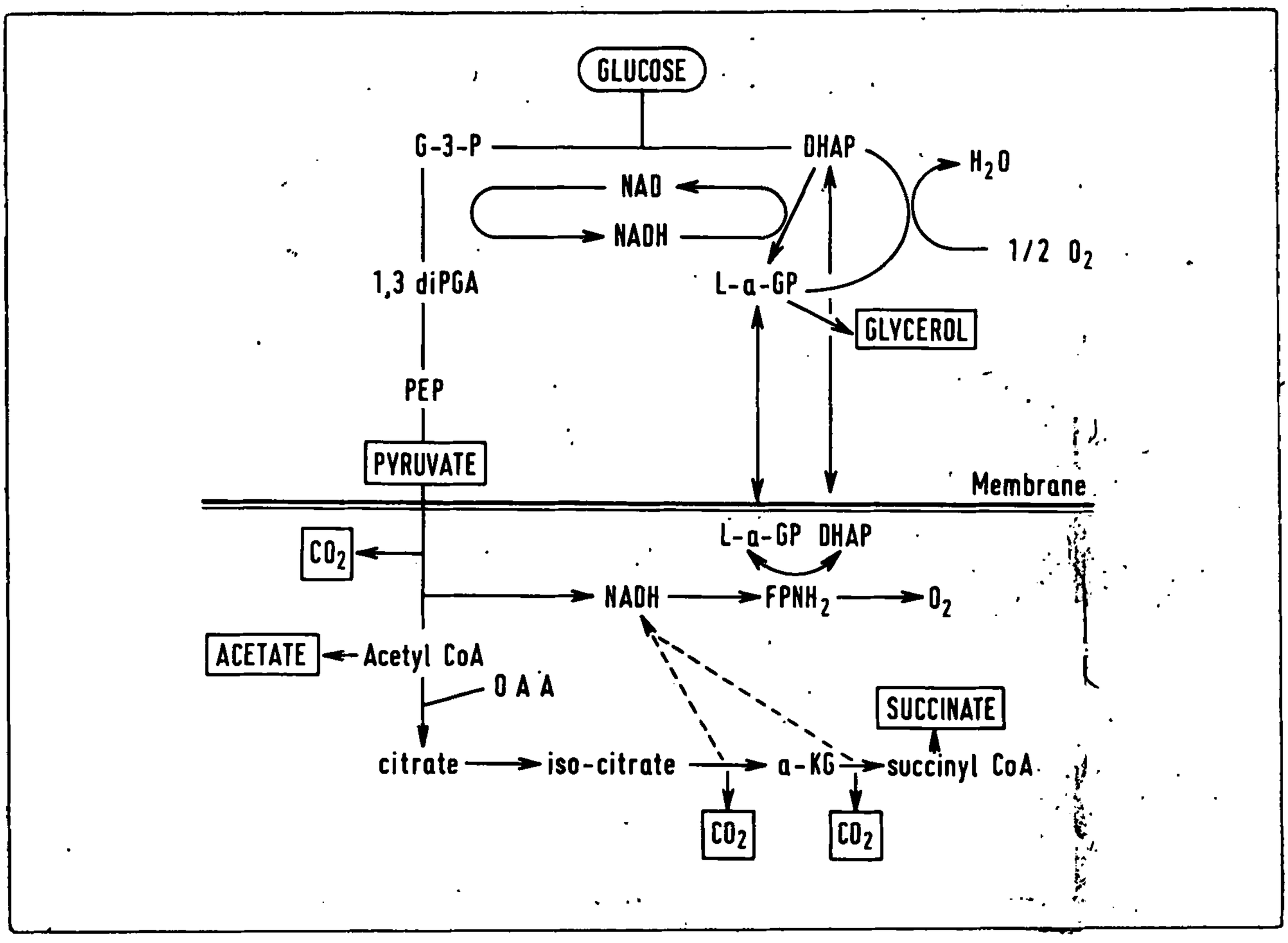


Plate 2. Metabolic pathways in T. rhodesiense EATRO 173.
The metabolism of the LS form is confined to the reactions above the horizontal division labelled "Membrane". The postulated metabolic developments in the SS form are depicted below this line. Metabolic end-products are shown in square enclosures.

presence in the SS forms of oxidative decarboxylation systems for pyruvate and ∝-oxoglutarate, and no further enzymic differentiation has become apparent. Succinate production *in vitro* by the pleomorphic strain is not likely to be via a reaction series involving CO_2 fixation, on the basis of the carbon balance studies. Furthermore, the *in vivo* significance of the partial tricarboxylic acid cycle which has been suggested as the route of formation of succinate is in some doubt as a result of the time-course studies.

The suggestion of Ryley (1962) that a lack of pyruvate oxidase prevents the further metabolism of pyruvate by the bloodstream trypanosomes, is invalid in the case of natural pleomorphic infections. The limiting enzymic factors instead appear to be citrate synthase and the succinoxidase system. Despite the capacity of the stumpy organisms to synthesise acetyl CoA, the fate of this complex appears to a large extent to be confined to the production of acetate, in conditions approaching those obtaining *in vivo*. A diagram of the metabolic capacities of LS and SS forms is shown in Plate 2.

2) EFFECTS OF MELAMINYL ARSENICALS ON CARBOHYDRATE METABOLISM

Experiments using lysed cells show that pyruvate oxidase is the primarily inhibited system at low concentrations of melarsen oxide. At a drug concentration of 10^{-5} M there is an increase in the ratio of μmoles pyruvate produced per μmole glucose utilised and a decrease in the gas exchange (Figure 8). The inhibition of oxygen utilisation appears to reflect the further metabolism of pyruvate as neither the glucose utilisation (Figure 8) nor the L-∝-GP oxidase (p. 79) are significantly inhibited at this concentration of melarsen oxide. In the lysed preparations hexokinase (I_{50} = 3 x 10^{-5} M ; Fairlamb, 1970) and pyruvate kinase (I_{50} = 10^{-4} M) are the next most sensitive sites.

It appears, however, that this sequence of sensitivities is not a reflection of the *in vivo* mode of action of the drug. It was not

possible to demonstrate the build-up of pyruvate from glucose in whole cells by selective inhibition of pyruvate oxidase, and in fact the primary effect is a decrease in the pyruvate yield (Figure 9). The glucose and oxygen utilisations and the evolution of CO_2 are inhibited in parallel over the range 1-60 x 10^{-7} M, whereas the yield of pyruvate per mole glucose decreases steadily (Figure 10). Evidence is presented to show a buildup of PEP at the expense of pyruvate in the presence of melarsen oxide (2×10^{-6} M) (Table 16) and pyruvate kinase by implication is thought to be the most sensitive enzyme in vivo to this trypanocide. The concomitant decrease in ATP production by this enzyme on inhibition, accounts for the rapid action of the drug.

The inhibition data enable some speculation to be made regarding the mode of glucose metabolism. Increasing melarsen oxide concentrations up to 2.3×10^{-5} M using cell lysates, increases the pyruvate yield per glucose but simultaneously decreases the carbon dioxide production. This CO_2 evolution appears to come then from the further metabolism of pyruvate; the inhibited reactions have a calculable RQ.of 2.02, equivalent to that of the pyruvate/acetate reaction. It is assumed that the inhibition of this reaction is by coupling of melarsen oxide with reduced lipoamide or with dihydrolipoate dehydrogenase, thus inhibiting the generation of reduced FAD/NAD and acetyl lipoate. Hence the exchange of oxygen and CO_2 is inhibited.

The first step in the oxidative decarboxylation of pyruvate by mammalian tissues is the formation of the TPP.CHOH.CH_3 complex with liberation of CO_2. Presumably this complex in the trypanosome is unable to break down spontaneously as CO_2 evolution is inhibited.

The data presented here are in agreement with those of Cantrell (1951,1953), who also demonstrated an inhibitory point between glucose-6-phosphate and pyruvate, showing a decrease in pyruvate production without

inhibition of glucose utilisation in whole cells of T. equiperdum. Chen (1948), however, used lysed preparations of the same organism to demonstrate that hexokinase is primarily inhibited by the arsenicals. This difference in behaviour between whole organisms and cell lysates is borne out by the present work (c.f. Figures 8 & 9). The data of Marshall (1948) which show that in T. evansi in vivo hexokinase is the point of inhibition probably do not indicate a difference between species. The high arsenical concentration (6×10^{-4} M) used by Marshall (1948) will obscure any primary inhibition of pyruvate kinase.

3) PYRUVATE KINASE

Despite the previously documented knowledge on the metabolism of slender T.rhodesiense, it has recently been stated (Webb, 1966 p.752) that "... trypanosomes are inactivated (by arsenicals) and killed because of a block in keto-acid oxidation", although Webb does criticise this statement. As far as is known the enzyme responsible for a net production of ATP in the metabolism of glucose by T.rhodesiense is pyruvate kinase. This enzyme would therefore make an ideal target for chemotherapeutic attack, an inhibition at this site eliminating the sole energy source of the organism. Evidence has been presented (in this thesis (pp. 78-85)) to show that this is in fact the primary site of action of melarsen oxide. Part II of this work has entailed a study of the trypanosome pyruvate kinase with a view to pin-pointing qualitative or quantitative differences from the host enzymes. Such studies may lead to the rational synthesis of more effective trypanocides.

Interaction with cations.

In common with all the pyruvate kinases which have so far been examined, the trypanosome enzyme exhibits a strict requirement for both

a univalent and a divalent cation. The role of the monovalent cation is still uncertain, but it has been suggested that it alters the enzymic conformation thus influencing the binding of substrates (Melchior, 1965). It is now widely accepted, however, that the divalent metal ion acts in conjunction with the nuclectide coenzyme, the $MgXDP^-$ species being the reactive complex.

Nucleotide specificity.

The nucleotide specificity of the parasite enzyme appears slightly different from that of the previously investigated mammalian enzymes, in that the K_M $MgGDP^-$ value is lower with respect to the K_M $MgADP^-$ value in the trypanosome system. This quantitative difference may reflect the high rate of protein synthesis in vivo in the trypanosome, the organisms having a generation time of 4 - 6 hours. Although no estimates of phosphotransferase activity have been made on the trypanosomal system, the apparent minimal activity of the tricarboxylic acid cycle, including the formation of GTP, makes an alternative source of this coenzyme a strict necessity assuming that this nucleotide is required for protein synthesis.

Stability and purification.

The stabilisation and purification procedures adopted for any enzyme are necessarily arbitrarily chosen and dependent upon both the source and nature of the enzyme. In this case, not only the enzyme activity but also its allosteric properties and interaction with inhibitors appear to be exceptionally labile. In contrast with the liver L isoenzyme (Tanaka et al., 1965) both substrate and coenzyme increased the rate of inactivation on storage, although dithiothreitol

protected both liver and trypanosome systems. The hydrophobic nature of the parasite enzyme is reflected in the stabilising power of glycerol, analagous to the effect of sucrose on the liver L isoenzyme (Rozengurt et al., 1969). Subsequent to purification, the instability of the parasite enzyme is similar to that of both liver isoenzymes on storage in the cold.

Interaction with PEP and FDP.

The sigmoidicity of the enzymic response to PEP is typical of, inter alia, an allosteric enzyme. No concrete significance may be placed on the absolute value of the Hill coefficient, n, as this figure may reflect either 1) the number of binding sites available for the substrate per enzyme molecule, or 2) the number of non-equilibrium configurations of the enzyme in the presence of the substrate. It is clear, however, that the substrate concentration does exert some control over the enzymic rate outwith the normal Michaelis response.

This activation of the enzyme by PEP is entirely dependent on the presence or absence of FDP. In the presence of this activating glycolytic intermediate, the homotropic substrate interactions are abolished, and plots of enzyme activity as a function of PEP concentration give the normal hyperbolic kinetic picture. A secondary effect of FDP is to increase the binding of PEP by the enzyme at high activator concentrations. This susceptibility to activation by a substance which is not catalytically active, is a fundamental characteristic of an allosteric enzyme, the activation being modulated by effector binding at a site other than the catalytic point. Also, the desensitisation of the enzyme to the homotropic PEP interactions on storage in the cold, indicates that the higher order kinetics obtained are due to the binding of more than one PEP molecule per enzyme molecule. This cold desensitisation has been attributed to a change in conformation, which is

dependent upon hydrophobic bond interactions, in the cases of pyruvate decarboxylase and homoserine dehydrogenase (Stadtman, 1966) and is thus in agreement with the stabilisation of the trypanosome PK by glycerol.

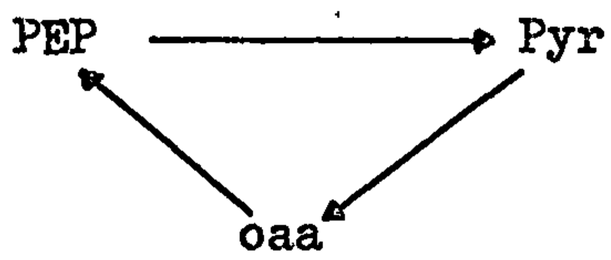
When the binding of FDP to the enzyme is investigated, two interesting characteristics of the system are found. Firstly extrapolation of the Hill coefficient for FDP to zero PEP concentration indicates only one binding site for the effector per enzyme molecule (n = 1) or multiple binding sites which are non-interacting. This is unusual in so far as the presence of a homotropic substrate effect is normally accompanied by a heterotropic reaction involving more than one effector molecule. In the case of a four sub-unit enzyme, consisting of two catalytic and two "control" sub-units, two molecules of effector may be bound. This may be the condition of the yeast PK system, although some doubt exists as to the subunit composition of this enzyme. Native enzyme molecular weights reported for yeast PK are uniformly in the range 160,000 - 200,000 (Kuczenski & Suelter, 1970; Bischofberger et al., 1970), but the sub-unit sizes obtained by ultracentrifugation differ in being 42,000 - 45,000 (Kuczenski & Suelter, 1970a) or 62,000 (Bischofberger et al., 1970). The work of these latter authors hence appears to indicate the possibility of an atypical trimeric system, which may apply to the trypanosome enzyme, in having one regulatory ($n_{FDP} = 1$) and two catalytic ($n_{PEP} = 2$) sub-units. However, Hill coefficients of n_{FDP} between 1.4 and 2.6 have been reported by other workers on the yeast enzyme (Hunsley & Suelter, 1969) and the situation in yeast is complicated by the dependence of FDP activation on the types and concentrations of activating cations employed in the enzyme assay. Also, n_{PEP} approaches 3.0 in this system at pH $\longrightarrow$ 7.0.

The second characteristic of the heterotropic FDP activation, is the synergism of the allosteric effector and the substrate in activating

the enzyme. At a high concentration of PEP ($> 5 \times 10^{-3}$ M), no effect of FDP is obtained, and likewise at a high concentration of FDP, no allosteric effect is produced by PEP. The allosteric effects induced by FDP and PEP are therefore synergistic. No change in the V_{max} value was found in the presence of FDP, and this enzyme is therefore classified as a "pure K" system in the terminology of Monod et al., (1965).

Significance of FDP activation.

The activity of pyruvate kinase in mammalian liver is controlled in two ways. The physiological and pathological status of the animal determines the hepatic enzyme concentration and the ratio of the isoenzymic forms present (Tanaka et al., 1967a,b), whereas the allosteric control of the L isoenzyme regulates the differentiation between glucogenic and glycolytic requirements. Thus both the rate and the direction of the glycolytic enzyme sequence may be influenced by controlling the activity of PK. The allosteric control of this L isoenzyme is mediated via a feed-forward activation by FDP (in the same manner as the trypanosome enzyme is activated), and via a feed-back inhibition by one of the enzymic products, ATP, and this activation and inhibition counteract each other. Hence in a condition where the hepatic cell has an excess of ATP, this coenzyme will inhibit pyruvate kinase and allow the glucogenic process to proceed. In the absence of this control, glucogenic PEP synthesised by the decarboxylation of oxaloacetate would immediately be dephosphorylated to pyruvate, thus promulgating a futile cycle of



In the trypanosomal enzyme system, such a situation may exist, as under strictly controlled conditions, little inhibition of this enzyme by ATP is apparent. However, it must be borne in mind that neither the glucogenic process *per se*, nor the storage of carbohydrate in any form, has been demonstrated in *T.rhodesiense*. The parasite, in its bloodstream form, is in an environment which ensures a constant extracellular supply of utilisable carbohydrate, and as such has no need to develop systems for either the synthesis or storage of such material. It may be argued that on transmission to the insect vector such systems would be advantageous, but the organism instead appears to compensate by the production of pathways which increase the efficiency of the utilisation of such carbohydrate as is available.

Trypanosomal glycolysis appears, then, to be permanently activated by the action of FDP on pyruvate kinase. The sigmoidal response of the enzyme to PEP itself may not be physiologically significant *in vivo*, but to decide this point, much more accurate data on the intracellular and compartmental concentrations of both PEP and FDP would be required. Some advance in this direction may come from a study of computer simulation of trypanosomal glycolysis.

Effect of melarsen oxide.

Although it is presumptuous to postulate a single site of action for a reaction as general as the one between an organic arsenical and a thiol compound, evidence has been produced in this work to indicate that the primary site of arsenical trypanocidal activity is pyruvate kinase. The apparent dichotomy between the evidence of Chen (1948) and Marshall (1948), who concluded that the trypanocidal action was due to inhibition of hexokinase, and the evidence of Cantrell (1953), who

states ".... these results convincingly rule out an action on hexokinase as the lethal action of mapharsen" is explicable in terms of the intracellular concentrations of the drug, as both PK and hexokinase are sensitive to the aromatic trivalent arsenicals, although the former enzyme appears to be inhibited at lower drug concentrations. The finding that PK activity is suppressed by these drugs also explains the inability of the organisms to survive utilising glycerol in the presence of the inhibitor.

The interaction of the enzyme with melarsen oxide at the molecular level is integrally connected with the binding of both PEP and FDP. The kinetic picture obtained with respect to $MgADP^-$ in the presence of the drug, shows typical non-competitive inhibition, with the K_M $MgADP^-$ unchanged and the V_{max} value decreased. The relationship between melarsen oxide inhibition and PEP concentration, however, is one of competition, as the V_{max} value remains unchanged and the S_{50}PEP value is increased in the presence of the arsenical under the available preincubation conditions. This competitive effect holds both in the unactivated system, and at all the concentrations of FDP used (up to 6.7×10^{-4} M), although significantly FDP also competes with melarsen oxide; the higher the activator concentration, the lower the inhibition obtained at a given concentration of drug. The tripartite system, then, consists of a synergistic competition between PEP and FDP to activate the enzyme, and an antagonistic competition between this activation and the inhibition by melarsen oxide. Significantly, the homotropic interaction between PEP molecules is unaltered by the presence of the arsenical. Unfortunately, these kinetic data do not indicate whether the drug is bound at the regulatory, the catalytic, or some other site.

The commercially available rabbit muscle PK is seen to be unaffected by the arsenical drug, and to have no heterotropic or homotropic allosteric

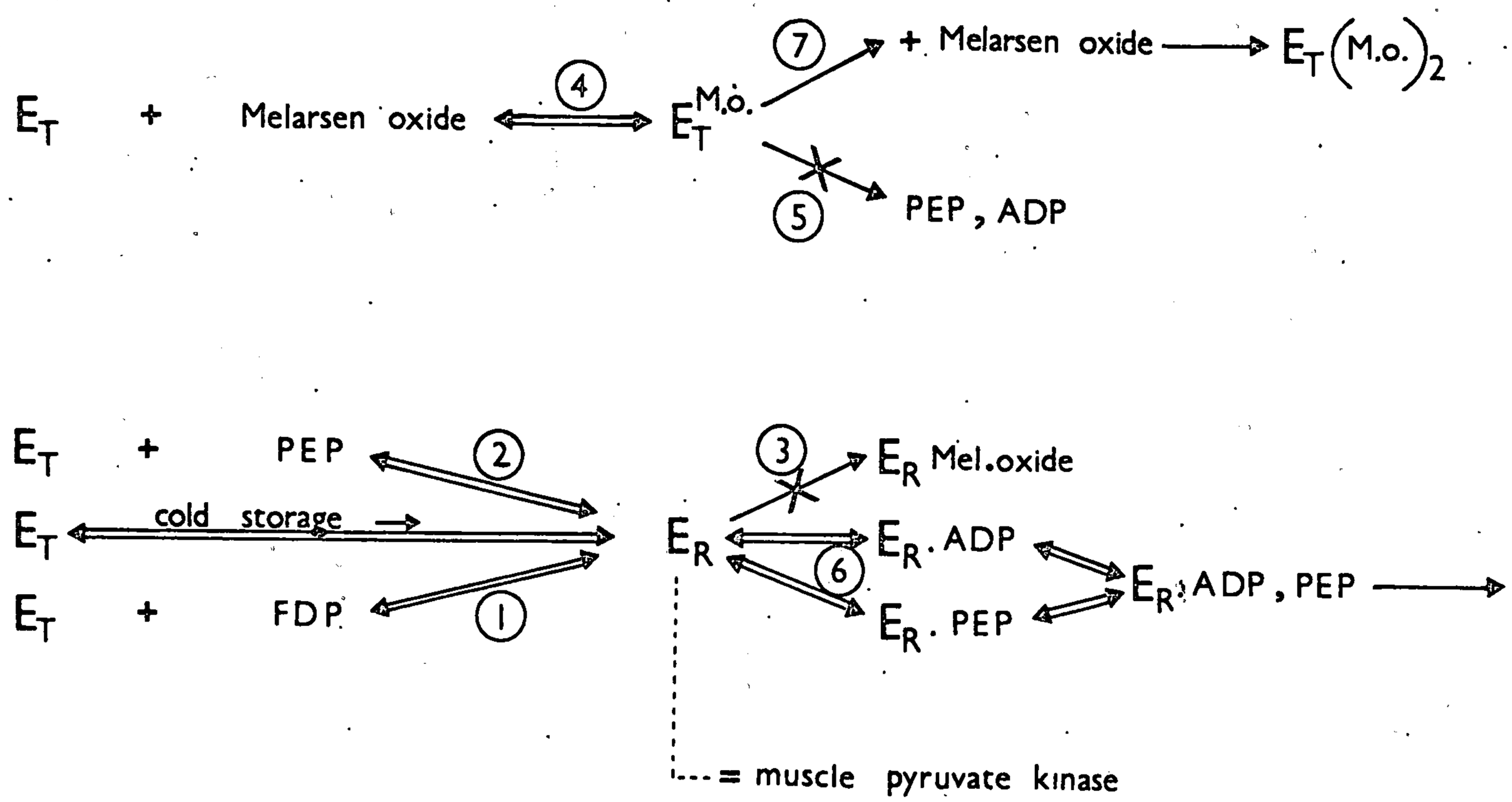


Fig. 43. A model to describe the interaction of trypanosome pyruvate kinase with substrate (PEP), coenzyme (ADP), activator (FDP) and an arsenical trypanocide (Melarsen oxide). The numbering of the reactions refers to the discussion of the model in the text.

properties. The response curve of activity to increasing PEP concentration is hyperbolic, with $n_{PEP} = 1$. This state is thus analagous to the trypanosome enzyme 1) after activation by FDP, or 2) in the presence of high PEP concentration. In the FDP activated state, $n_{PEP} \longrightarrow 1$ in the parasite system, and in high concentrations of PEP, the latter part of the sigmoidal v versus s plot approaches a hyperbolic shape. This is reflected in the thecretically sigmoidal presentation of the Hill plot; at sufficiently high concentrations of the substrate, the allosteric site may be regarded as saturated, and the kinetic association/dissociation only involves the catalytic site. In either of these two conditions of the trypanosome enzyme, where $n_{PEP} \longrightarrow 1$, the melarsen oxide inhibition decreases and approaches zero in a competitive fashion with increasing PEP and FDP. Thus the muscle PK is analagous to the trypanosome enzyme in these states, both in the response to PEP and in the insensitivity to melarsen oxide.

A model consistent with these findings for the trypanosome and muscle enzymes may be built as shown in Figure 43. In solution in the absence of drug, substrate or modifier, the enzyme can exist in two conformations in equilibrium, the equilibrium lying greatly in favour of a catalytically inactive (E_T) form. The addition of FDP (1) or PEP (2) shifts this equilibrium by combination of the effector with the enzyme at an allosteric site, to give the catalytically active E_R configuration (E_{PEP} or E_{FDP}). At a high concentration of either PEP or FDP, the equilibrium will then be shifted grossly to favour the E_R type enzyme and hyperbolic kinetics with respect to PEP concentration will follow. This enzyme configuration is therefore analagous to the one in which the muscle PK is "fixed", and cannot bind, or be inhibited by, melarsen oxide (3). Prolonged storage in the cold has the effect of altering, perhaps by variation of the hydrophobic bonds of the protein, the position of

equilibrium between E_T and E_R, and spontaneously gives rise to the E_R state, thus decreasing the "interaction coefficient" of the PEP binding sites.

The arsenical drug may therefore be competing, not for a site on the enzyme, but for a specific conformation of the enzyme, E_T (4). Once melarsen oxide is bound, however, no catalytic activity remains, as 100% inhibition may be obtained (5). As the inhibition is competitive with both PEP and FDP, this reaction between enzyme and drug must be reversible. The diagram also shows the random bireactant nature of the enzymic mechanism, as deduced from the mutual independence of the K_M and S_{50} values for $MgADP^-$ and PEP respectively, and may be described as a Random Bi-Bi type reaction in the terminology of Cleland (1963).

Some support is given to this model by the findings on the other mammalian PKs investigated. Thus both liver isoenzymes are allosterically activable, and both are melarsen oxide sensitive, whereas the enzymes from heart and brain are presumably "fixed" in the E_R state in the same manner as the muscle enzyme, show no allosteric properties, and are unaffected by the arsenical.

The trypanosome enzyme, however, differs in two ways from the most similar host enzyme, the liver L isoenzyme. Firstly the liver PK is inhibited by ATP i.e. this modifier reverses the $E_T \longrightarrow E_R$ transition brought about by FDP. Secondly, variation of the pH of the enzymic assay over the range 6.0 - 8.0 does not affect the interaction between PEP binding sites on the parasite enzyme, the Hill coefficient being invariable over this range. The value of n_{PEP} for the liver enzyme, however rises from n = 1 at pH = 6.8 to n = 3 at pH 8.5 (Rozengurt et al., 1969). Thus the $E_T \longrightarrow E_R$ transition in the latter case may be effected by an increase in the H^+ ion concentration, whereas this is not the case with the trypanosome system.

The question arises, however, as to how low concentrations of a purely competitive inhibitor can act as such an effective trypanocide. It may have been expected that in the presence of melarsen oxide, glycolysis would continue to produce PEP to a level at which the buildup could overcome the competitive inhibitory effect, and the organism would continue to function at an elevated intracellular PEP concentration.

This is improbable for three reasons. Firstly, as previously stated, hexokinase is sensitive to melarsen oxide, and the glycolytic flux will consequently be diminished. Secondly, the trypanosome has the property of concentrating the arsenicals from dilute solution (c.f. e.g. Hawking 1937, 1938) leading to a cell/medium partition ratio of $5 - 10 \times 10^3$. The third, and most pertinent explanation arises from the studies on the PK from liver. Under the limited preincubation conditions available due to the instability of the parasite enzyme, similar kinetics are obtained with both liver isoenzymes i.e. competition between PEP and the drug. However, elongation of the preincubation period of drug + enzyme changes the kinetic analysis to a non-competitive inhibition. This may be due to either the slow reaction of E_T with a second molecule of melarsen oxide (as depicted (7) in Figure 43) or a time dependent irreversible change in the configuration of the enzyme after binding one melarsen oxide moiety. Assuming that this occurs with the trypanosomal enzyme in vivo, a reasonable explanation of the arsenical toxicity follows. It may be mentioned that such a time-dependent kinetic change has previously been noted for xanthine oxidase inhibition by arsenite (Peters & Sanadi, 1961).

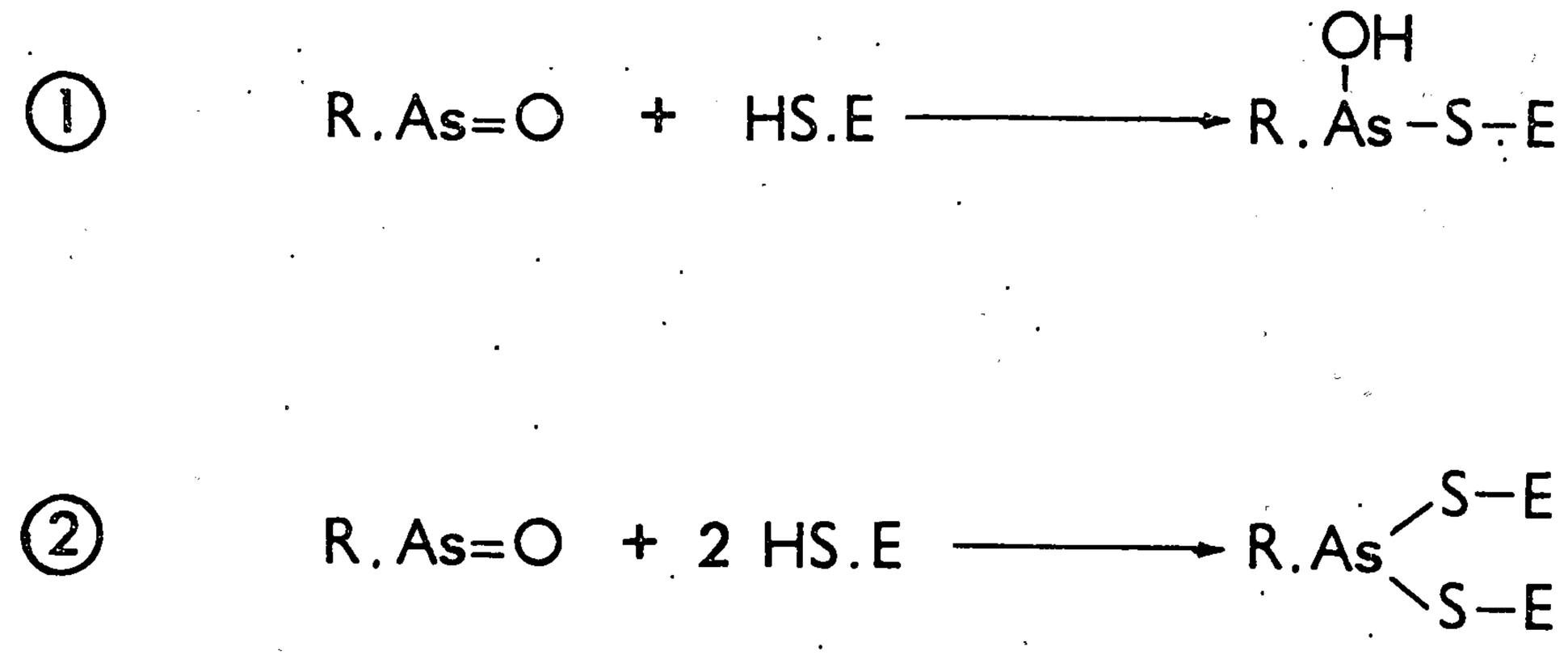


Fig. 44. The possible modes of interaction between a mono-substituted trivalent arsenical and a thiol enzyme.

Mechanism of melarsen oxide binding to pyruvate kinase.

The primary competitive effect between PEP and melarsen oxide also gave rise to speculation as to the nature of the bond between drug and enzyme. Melamine itself has no inhibitory effect upon the enzyme, (Table 23) and hence the active constituent appears, as postulated, to be the arsenoxide grouping. Whereas arsenite is known to affect some enzymes in its capacity as a non-specific anion, no evidence has been produced for this type of action in the case of aromatic arsenicals. Again, inorganic arsenite may act as a reductant and thus inhibit an enzymic process, but the stimulation of trypanosomal PK by a strong reducing agent such as dithiothreitol argues strongly against this as a mechanism for melarsen oxide inhibition. Hence the conclusion must be that this drug exerts its inhibitory effect by virtue of the interaction of the trivalent arsenic atom with an enzymic thiol group, or groups.

The three types of interaction between a trivalent monosubstituted arsenical and a thiol enzyme are shown in Figure 44. Of these three reactions it has long been assumed that reaction III is the mechanism by which potent inhibition occurs. Reaction II appears unlikely due to the steric difficulty in binding two enzyme molecules at the fixed arsenic bond angle. Reaction I has been dismissed due to the inherent instability of the product relative to the tight binding in the form of the cyclic thioarsinite (III), and no evidence has previously been put forward for the production of this arsinous acid type complex between an enzyme and an aromatic arsenical. In fact the relative susceptibility of an enzyme to monosubstituted (R-As=O) and disubstituted (R_2=As-Cl) arsenicals has been used since the work of Lotspeich and Peters (1951) as a criterion for the determination of whether the enzyme

contains a catalytically required dithiol or monothiol grouping respectively. This method of distinction, however, leaves much to be desired, as, for example, there is no apparent reason why a compound of the R_2 = As--Cl form should not react equally well with one of the two --SH groups in a dithiol enzyme.

The two major observations which must be accounted for in the case of the interaction of PK with arsenicals are 1) the competitive nature of the primary inhibition by melarsen oxide and 2) the insensitivity of the enzyme to inorganic arsenite. If the primary reaction between the enzyme and melarsen oxide were of type III (Figure 44) the cyclic thioarsinite formed should be stable enough to resist any competition by PEP i.e. it should be an irreversible inactivation of the enzyme. Hence it is postulated that the competitive inhibition of the enzyme is due to the formation of a readily hydrolysable type I compound, and that the time-dependent change to non-competitive inhibition is due to the formation of a stable type III complex in which the enzyme is fixed in a normally unstable conformation. Type II complexes in this case may be eliminated by the observation that the Hill coefficient for melarsen oxide = 1.0. The inability of the pentavalent arsenicals to inhibit the trypanosomal enzyme, and the potent action of pCMB, lend strength to the interpretation of the data in terms of a thiol binding reaction.

The differential inhibitory power of inorganic arsenite and the organic arsenicals has been observed previously in diverse enzymic systems e.g. succinoxidase (Barron and Singer, 1945) and aconitase (Peters, 1955). Arsenite has practically no affinity for simple monothiols: Drummond and Stern (1960) observed no mercaptide formation between arsenite and gluta thione even at 100 mM arsenite. It does, however, react readily with simple dithiols such as dimercaprol (BAL; Aldridge and Cremer, 1955). Similarly it has been reported that from a mixture of arsenite and

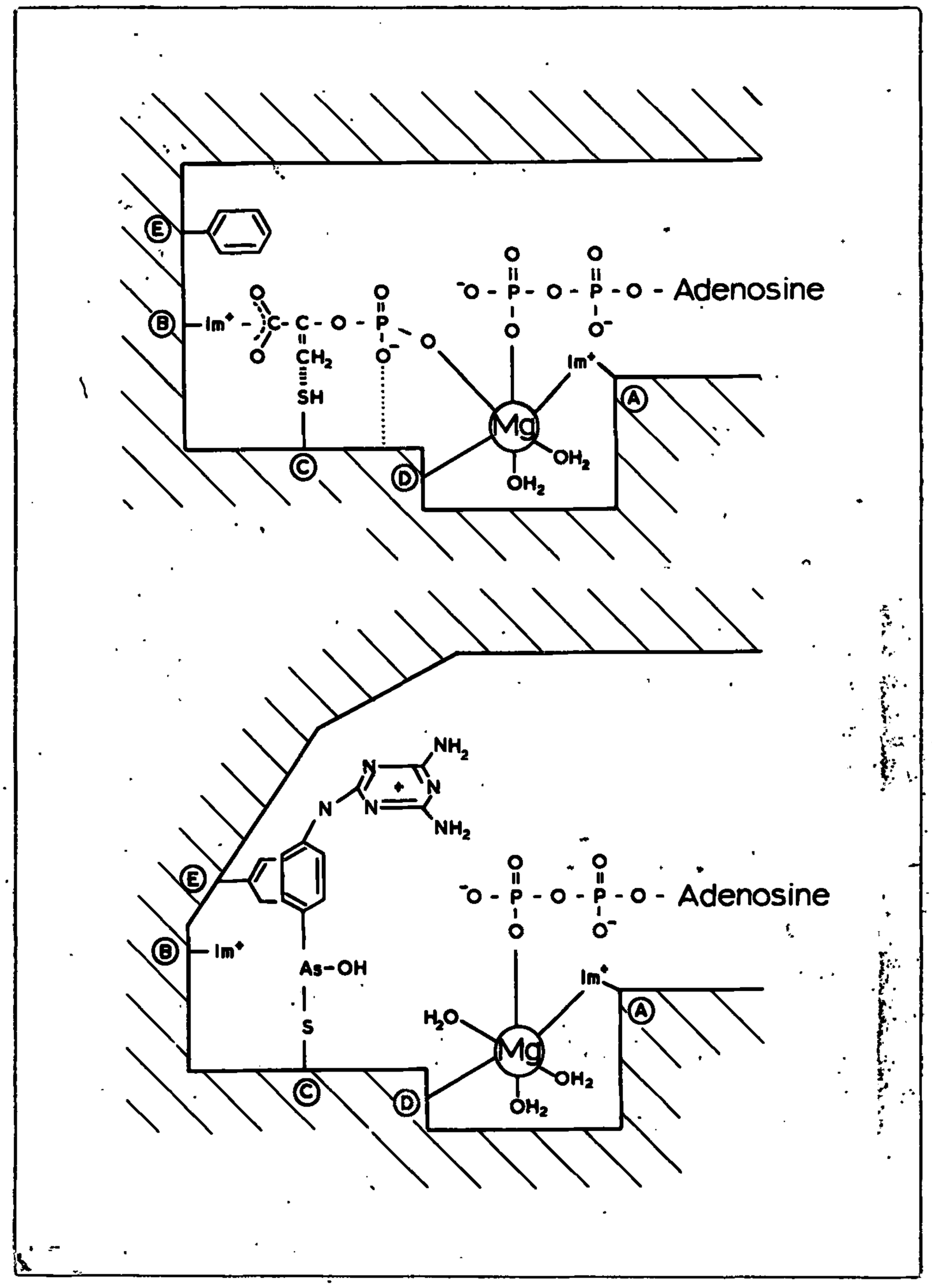


Plate 3. The interaction of trypanosome pyruvate kinase with ADP, PEP and melarsen oxide.

PEP is bound at the catalytic site in the allosteric E_R conformation. (above). Melarsen oxide competes for, and can only bind to, the E_T conformation (below). The nature and significance of the enzymic groups labelled A - E are discussed in the text.

cysteine, it is not possible to isolate the monothioarsinite as the instantaneous product is the dithioarsinite. Thus it appears that for interaction between arsenite and an enzyme, there may be a strict requirement for a vicinal dithiol group to produce inhibition. It is significant that in no case where an enzyme has been categorised as a dithiol protein has there been shown a differential inhibition between arsenite and the monosubstituted organic arsenicals.

This observation then lends strength to the hypothesis that the primary and necessary reaction between melarsen oxide and trypanosomal PK concerns a single -SH group on the enzyme. The trypanocidal effect follows as a result of secondary changes in the protein structure brought about after binding the melarsen oxide in the form of a relatively unstable monothioarsinite, and these secondary changes enable the formation of a relatively stable cyclic dithioarsinite to occur.

Substrate and inhibitor binding to the active site of pyruvate kinase.

The preferred model which has been presented for the action of melarsen oxide relies upon competition between drug and substrate for an enzymic configuration rather than for a specific binding site. Certain speculations may be made, however, on the basis of a previously described mechanism for the enzyme. Mildvan and Cohn (1966) correlated the enzyme reaction kinetics with proton relaxation rate studies to deduce the structure of the ternary intermediate complex between PK, PEP, ADP and the divalent cation. Plate 3 is based in part on the work of these authors.

The binding of ADP to trypanosome PK involves a group in the catalytic site which dissociates with a pK_a of 6.6 (page 116). This pK_a region is typical of a histidyl residue and is shown as group A (Plate 3). The binding of ADP has also been shown to be independent of the presence of melarsen oxide.

The data on the effect of pH on the pS_{50} value for PEP (page 119) combined with the titration characteristics of PEP indicate the necessity for a doubly dissociated phosphate group on this substrate, the pK_a of the ionising group being in the region of pH 5.9-6.1. This required ionisation may be involved in ligand formation with the divalent cation. The other dissociable group involved in PEP binding has been shown to be an enzymic group with a pK_a of 6.7-7.0. Again this is typical of an imidazole group dissociation, and this group is probably involved in binding the nucleophilic carboxyl group of PEP (group B, Plate 3). It is then conceivable that the proximity of the charged substrate group will elevate the pK_a value of this imidazole dissociation and thus the possibility that groups (3) and (4) on page 121 are identical would be validated.

The function of group C is the protonation of the substrate vinyl group, converting it to a $-CH_3$ group and effecting the enol-keto tautomerisation of the substrate. Unfortunately investigation of the pH variation of the kinetic parameters of the trypanosome PK could not be extended into the region of thiol dissociation (pH 8.5 - 9.0). However, the same technique has been used to demonstrate the involvement of a thiol residue (pK_a = 9.0) in the binding of PEP to the PK of S. carlsbergensis, another allosterically controlled enzyme (Wieker and Hess, 1971). A thiol group at this position, perhaps hydrogen bonded to a histidyl residue as postulated by Watts and Rabin (1962) for creatine kinase, would have the required acid-base properties to effect the protonation.

The monothiol/melarsen oxide interaction previously described could conceivably occur with this cysteine residue (Plate 3). The second thiol, involved in the rate-limiting formation of the stable, cyclic dithio-arsinite may not be situated directly in the active site, although the

second divalent cation ligand to the protein has been suggested to involve a thiol group, D (Mildvan and Cohn, 1965).

Stabilisation of the monothioarsinite by interaction of the phenyl ring, and perhaps of the melaminyl residue, with protein groups, would account for the inactivity of arsenite as an inhibitor. This will probably occur via an apolar (hydrophobic) interaction with a phenylalanyl or other aromatic side-chain (group E). The requirement for this temporary stabilisation could not be met in the case of inorganic arsenite which would therefore be unable to produce the stable, cyclic thicarsinite.

Thus, in the unactivated E_T state the thiol will be accessible for the binding of melarsen oxide. Binding of PEP or FDP to an allosteric site induces a conformational change at the catalytic site and may simply sterically prohibit the binding of melarsen oxide. The interaction of trypanosome PK with the arsenicals may now be described by a combination of Figs. 43, 44 and Plate 3. It must be emphasised however, that while the kinetic evidence outlined in this work supports this model, these mechanistic speculations are solely intended as a working hypothesis on which to base further investigation.

4) THE IN VIVO MECHANISM OF THE TRYPANOCIDAL EFFECTS OF MELARSEN OXIDE.

It is recognised that in vitro experimentation to determine the affinity of a drug for enzymic systems may be misleading. However a combination of the whole cell carbon balance data and the proven susceptibility of trypanosome PK for melarsen oxide do indicate that this is a feasible mechanism for the trypanocidal action of the drug. The lack of effect of melamine in this system and the comparable activities of melarsen oxide, phenylarsenoxide and p-aminophenylarsenoxide

indicate that the evolution of the drug into its present state has not markedly affected its toxicity at its site of action, but has probably served to increase the differential uptake of the arsenical by parasite and host cells. This is demonstrated by the higher sensitivity of the liver enzymes to the drug, and thus in the absence of a differential permeability factor, little therapeutic benefit would be obtainable from this series of compounds. One further piece of evidence supporting PK as a plausible site of action of melarsen oxide and the related arsenicals, was obtained by Eagle et al., in 1946. These authors demonstrated that when trypanosomes are incubated in phenylarsenoxide (0.013 mM) they are totally immobilised after approximately 1-2 minutes. If, 5-10 minutes after addition of the drug, a high concentration of a thiol compound is added, the arsenical is partially withdrawn from the cells and motility is totally regained by the organisms. Cysteine, for example, decreased the cell/medium drug ratio from 150 to 30, and dimercaprol had a similar effect. Thus, although the organisms still contained a 30 fold excess of the arsenical over the solution, no trypanocidal effect was found. It appears then that in this case the primary effect of the arsenicals is again easily reversible, and the more weakly bound fraction of the arsenical is the effective trypanocide, as would be the case were PK the focal point of attack. Significantly, cysteine cannot normally reverse the binding of phenylarsenoxide to lipoate in this fashion.

If the time lapse between addition of the drug and the thiol is increased to over 25 minutes, complete reversal is not obtained. However, although this may reflect the secondary, irreversible reaction of the drug with PK, after such a period some cell lysis may occur.

5) CONCLUSION.

The pleomorphism associated with the bloodstream forms of wild type T.rhodesiense appears to have a biochemical as well as a morphological significance, although the in vivo pathways of glucose metabolism are not as greatly different between the slender and stumpy organisms as was originally postulated. The capacity to synthesise acetate via acetyl CoA from pyruvate is the most significant development which takes place in the slender/stumpy transformation. As the time course carbon balance data show, whatever the mechanism of succinate formation may be, its significance in vivo must be doubtful. However, the development of this system does not seem to be adequate to explain the dependence of cyclical transmission upon the presence of short stumpy organisms in a bloodstream infection, as the further development of e.g. the succinoxidase system, is necessary to promote full operation of the tricarboxylic acid cycle. Although it has been tacitly assumed since the work of von Brand (1959) and Ryley (1962), that the energy conserving metabolism of the cultured epimastigote is via this cycle and the cytochrome chain, it should be considered whether or not carbohydrate is the in vivo substrate. In the environment of the tsetse fly midgut after a blood meal, the supply of exogenous carbohydrate would very quickly be exhausted and the metabolic switch may not be from glycolysis ⟶ tricarboxylic acid cycle but from carbohydrate ⟶ amino acid metabolism. This problem deserves further investigation.

When one considers the production of ATP by the bloodstream organisms, the total dependence of the organism upon glycolytic production of this compound appears enigmatic. The intense motility of the organisms and the magnitude of the division rate with concomitant synthetic requirements, would suggest the inadequacy of this system. The observations on the ADP dependence of pyruvate oxidation and the possibility

of the presence of an alternative oxygen utilising system to the L-α-glycerophosphate oxidase may indicate that the trypanosome is not totally dependent upon the glycolytic system. The second of these possibilities is however to a large extent eliminated by the major hypothesis presented in this thesis, that PK is the active site of arsenical trypanocidal activity.

The action of this drug has been defined by its inhibitory action on glycolytic ATP production and the mechanism of action has been investigated on a purified enzymic system. On the justifiable assumption that the reaction involves enzymic thiol groups, a mechanism of action of the drug has been evaluated in molecular terms. The variation of the enzymic kinetic parameters with pH, carried out in an attempt to show the involvement of an -SH group or groups in the enzymic mechanism was unsuccessful due to the total instability of the enzyme and the exceedingly low rates of activity in the expected ionisation range of thiol groups (8.3 - 8.6, Benesch & Benesch, 1955).

The pyruvate kinase of the trypanosome has been characterised, and much evidence presented to show that this is an allosteric system. The allosteric properties appear to be integrally connected with the action of the arsenical drugs, and this hypothesis has been briefly extended to some host PKs. The parasite enzyme is allosterically activated by FDP and PEP and these properties have been correlated with the glycolytic requirements of the organism and compared to the functions of the liver isoenzymes.

From the point of view of rational chemotherapy, it would appear that the arsenicals do not depend entirely on their permeability characteristics to produce their specific action. The inability of these drugs to inhibit the non-allosteric pyruvate kinases of such host organs as heart, brain and skeletal muscle indicates that the possession

by the parasite of an allosteric PK is instrumental in allowing the arsenicals to exert their chemotherapeutic effect. Alteration of the substituents on the phenylarsenoxide ring will, however, have a secondary effect on the penetration of the drugs to the allosteric host PKs such as those of liver. Further extension of the work on the interaction of melarsen oxide and its analogues with trypanosome PK should therefore be accompanied by an investigation of the characteristics of the interaction between the drug and mammalian ∝-keto acid oxidases (the primary site of inhibition in the host) and of the specific uptake of the drug by liver and trypanosomes. It is nevertheless of prime importance to determine the site and mechanism of action of a therapeutically used compound, as a basis for further improvement. Development of further organic arsenicals for use against trypanosomiasis may now be based on the knowledge that one of the major activities of these drugs is the inhibition of pyruvate kinase.

The other major contribution of this work is in the question of the position of monomorphic strains as experimental organisms. Most screening tests are carried out on old laboratory strains which have acquired certain stable characteristics. The increased virulence and the loss of pleomorphism in these strains, associated in some cases with an increased sensitivity to drugs, may have been thought to give a misleading impression of the therapeutic value of the drugs. However, although biochemically different from the natural organisms, the mode of action of at least the arsenical series appears to be applicable to the monomorphic strains, to the same extent. The convenience involved in the use of such strains therefore appears justifiable in terms of the interpretation of the arsenical action.

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